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(54) Title: METHODS AND COMPOSITIONS FOR PEPTIDE SYNTHESIS

(57) Abstract

The present invention relates, first, to methods for the synthesis of peptides, in particular T-20 (also referred to as "DP-178"; SEQ ID NO:1) and T-20-like peptides. Such methods utilize solid and liquid phase synthesis procedures to synthesize and combine groups of specific peptide fragments to yield the peptide of interest. The present invention further relates to individual peptide fragments which act as intermediates in the synthesis of the peptides of interest (e.g., T-20). The present invention still further relates to groups of such peptide intermediate fragments which can be utilized together to produce full length T-20 and T-20-like peptides.

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METHODS AND COMPOSITIONS FOR PEPTIDE SYNTHESIS

1. INTRODUCTION

The present invention relates, first, to methods for the synthesis of peptides, in particular T-20 (also referred to as "DP-178"; SEQ ID NO:1) and T-20-like peptides. Such methods utilize solid and liquid phase synthesis procedures to synthesize and combine groups of specific peptide fragments to yield the peptide of interest. The present invention further relates to individual peptide fragments which act as intermediates in the synthesis of the peptides of interest (e.g., T-20). The present invention still further relates to groups of such peptide intermediate fragments which can be utilized together to produce full length T-20 and T-20-like peptides. The present invention still further relates to methods for the purification of peptides, in particular T-20 and T-20-like peptides, and the individual peptide fragments which act as intermediates in the synthesis of the subject peptides.

2. BACKGROUND

Recently, a large number of peptides have been identified which exhibit an ability to inhibit fusion-associated events, and, importantly, also exhibit potent antiviral activity. See, for example, U.S. Patent Nos. 5,464,933; 5,656,480 and PCT Publication No. WO 96/19495T-20. As these peptides to extensively be used, as therapeutics, for example, the need arises for an ability to synthesize in large scale quantities.

While techniques exist for peptide synthesis, (see, e.g., Mergler et al., 1988, Tetrahedron Letters 29:4005-4008; Mergler et al., 1988, Tetrahedron Letters 29:4009-4012; Kamber et al. (eds), "Peptides, Chemistry and Biology, ESCOM, Leiden, 1992, 525-526; and Riniker et al., 1993, Tetrahedron Letters 49:9307-9320) no techniques currently exist which can

be utilized for large scale, economical production of easily purified peptides such as T-20 and T-20-like peptides.

3. SUMMARY OF THE INVENTION

The present invention relates, first, to methods for the synthesis of peptides, in particular T-20 (also referred to as "DP-178"; SEQ ID NO:1) and T-20-like peptides. Such methods utilize solid and liquid phase synthesis procedures to synthesize and combine groups of specific peptide fragments to yield the peptide of interest. Generally, the methods of the invention comprise synthesizing specific side-chain protected peptide fragment intermediates of T-20 or a T-20-like peptide on a solid support, coupling the protected fragments in solution to form a protected T-20 or T-20-like peptide, followed by deprotection of the side chains to yield the final T-20 or T-20-like peptide. A preferred embodiment of the methods of the invention involves the synthesis of a T-20 peptide having an amino acid sequence as depicted in SEQ ID NO:1.

The present invention further relates to individual peptide fragments which act as intermediates in the synthesis of the peptides of interest (e.g., T-20). The peptide fragments of the invention include, but are not limited to, those having amino acid sequences as depicted in Table 1 below:

TABLE 1

PEPTIDE NO.	AMINO ACID SEQUENCE	CORRESPONDING NUMBERED AMINO ACID SEQUENCE OF T-20
1	YTSLIHSL (SEQ ID NO:2)	1-8
2	YTSLIHSLIEESQNQ (SEQ ID NO:3)	1-15
3	YTSLIHSLIEESQNQQ(SEQ ID NO:4)	1-16
4	YTSLIHSLIEESQNQQEK (SEQ ID NO:5)	1-18

PEPTIDE NO.	AMINO ACID SEQUENCE	CORRESPONDING NUMBERED AMINO ACID SEQUENCE OF T-20
5	IEESQNQ (SEQ ID NO:6)	9-15
6	IEESQNQQ (SEQ ID NO:7)	9-16
7	QEKNEQELLELDKWASLWNW (SEQ ID NO:8)	16-35
8	QEKNEQELLELDKWASLWNWF (SEQ ID NO:9)	16-36
9	EKNEQEL (SEQ ID NO:10)	17-23
10	EKNEQELLEL (SEQ ID NO:11)	17-26
11	EKNEQELLELDKWASLWNWF (SEQ ID NO:12)	17-36
12	NEQELLELDKWASLWNW (SEQ ID NO:13)	19-35
13	NEQELLELDKWASLWNWF (SEQ ID NO:14)	19-36
14	LELDKWASLWNW (SEQ ID NO:15)	24-35
15	LELDKWASLWNWF (SEQ ID NO:16)	24-36
16	DKWASLWNW (SEQ ID NO:17)	27-35
17	DKWASLWNWF (SEQ ID NO:18)	27-36
18	EKNEQELLELDKWASLWNW (SEQ ID NO:19)	17-35

The present invention still further relates to particular groups of peptide fragments which act as intermediates in the synthesis of the peptide of interest. The groups of peptide fragments according to the invention include Groups 1-20, as designated in Table 2 below.

TABLE 2

Group	Amino Acid Sequence		Corresponding Numbered Amino Acid Sequence of T-20
1	YTSLIHSLIEESQNQQ	(SEQ ID NO:4)	1-16
	EKNEQELLELDKWASLWNWF	(SEQ ID NO:12)	17-36
2	YTSLIHSLIEESQNQQ	(SEQ ID NO:4)	1-16
	EKNEQELLEL	(SEQ ID NO:11)	17-26
	DKWASLWNWF	(SEQ ID NO:18)	27-36
3	YTSLIHSLIEESQNQQ	(SEQ ID NO:4)	1-16
	EKNEQELLEL	(SEQ ID NO:11)	17-26
	DKWASLWNW	(SEQ ID NO:17)	27-35
4	YTSLIHSL	(SEQ ID NO:2)	1-8
	IEESQNQ	(SEQ ID NO:6)	9-15
	EKNEQELLELDKWASLWNWF	(SEQ ID NO:12)	17-36
5	YTSLIHSL	(SEQ ID NO:2)	1-8
	IEESQNQ	(SEQ ID NO:6)	9-15
	EKNEQELLEL	(SEQ ID NO:11)	17-26
	DKWASLWNWF	(SEQ ID NO:18)	27-36
6	YTSLIHSL	(SEQ ID NO:2)	1-8
	IEESQNQ	(SEQ ID NO:6)	9-15
	EKNEQELLEL	(SEQ ID NO:11)	17-26
	DKWASLWNW	(SEQ ID NO:17)	27-35
7	YTSLIHSL	(SEQ ID NO:2)	1-8
	IEESQNQQ	(SEQ ID NO:7)	9-16
	EKNEQELLELDKWASLWNWF	(SEQ ID NO:12)	17-36
8	YTSLIHSL	(SEQ ID NO:2)	1-8
	IEESQNQQ	(SEQ ID NO:7)	9-16
	EKNEQELLEL	(SEQ ID NO:11)	17-26
	DKWASLWNWF	(SEQ ID NO:18)	27-36
9	YTSLIHSL	(SEQ ID NO:2)	1-8
	IEESQNQQ	(SEQ ID NO:7)	9-16
	EKNEQELLEL	(SEQ ID NO:11)	17-26
	DKWASLWNW	(SEQ ID NO:17)	27-35

Group	Amino Acid Sequence		Corresponding Numbered Amino Acid Sequence of T-20
10	YTSLIHSLIEESQNQQ	(SEQ ID NO:4)	1-16
	EKNEQEL	(SEQ ID NO:10)	17-23
	LELDKWASLWNWF	(SEQ ID NO:16)	24-36
11	YTSLIHSLIEESQNQQ	(SEQ ID NO:4)	1-16
	EKNEQEL	(SEQ ID NO:10)	17-23
	LELDKWASLWNW	(SEQ ID NO:15)	24-35
12	YTSLIHSL	(SEQ ID NO:2)	1-8
	IEESQNQ	(SEQ ID NO:6)	9-15
	EKNEQEL	(SEQ ID NO:10)	17-23
	LELDKWASLWNWF	(SEQ ID NO:16)	24-36
13	YTSLIHSL	(SEQ ID NO:2)	1-8
	IEESQNQ	(SEQ ID NO:6)	9-15
	EKNEQEL	(SEQ ID NO:10)	17-23
	LELDKWASLWNW	(SEQ ID NO:15)	24-35
14	YTSLIHSL	(SEQ ID NO:2)	1-8
	IEESQNQQ	(SEQ ID NO:7)	9-16
	EKNEQEL	(SEQ ID NO:10)	17-23
	LELDKWASLWNWF	(SEQ ID NO:16)	24-36
15	YTSLIHSL	(SEQ ID NO:2)	1-8
	IEESQNQQ	(SEQ ID NO:7)	9-16
	EKNEQEL	(SEQ ID NO:10)	17-23
	LELDKWASLWNW	(SEQ ID NO:15)	24-35
16	YTSLIHSLIEESQNQ	(SEQ ID NO:3)	1-15
	QEKNEQELLELDKWASLWNWF	(SEQ ID NO:9)	16-36
17	YTSLIHSLIEESQNQ	(SEQ ID NO:3)	1-15
	QEKNEQELLELDKWASLWNWF	(SEQ ID NO:9)	16-36
18	YTSLIHSLIEESQNQQEK	(SEQ ID NO:5)	1-18
	NEQELLELDKWASLWNWF	(SEQ ID NO:14)	19-36
19	YTSLIHSLIEESQNQQEK	(SEQ ID NO:5)	1-18
	NEQELLELDKWASLWNW	(SEQ ID NO:13)	19-35
20	YTSLIHSLIEESQNQQ	(SEQ ID NO:4)	1-16
	EKNEQELLELDKWASLWNW	(SEQ ID NO:19)	17-35

This invention is based, in part, on the inventors' unexpected discovery that certain combinations of solid phase liquid phase synthetic reactions allow high purity T-20 and T-20-like peptides to be manufactured for the first time on a large scale with high throughput and high yield. In particular, in accordance with the methods of the invention, T-20 and T-20 peptides may be synthesized on a scale of one or more kilograms. It has been found that by selecting the specific T-20 peptide fragments of the invention for solid phase synthesis, the highly efficient coupling of solid phase techniques may be exploited without having to use the 3-, 4- or even 5-fold excess of amino acids and reagents that are normally required in solid phase synthesis. The methods of the invention use only about a 1.5-fold of amino acid in the solid phase synthesis of the peptide fragments of the invention. This cost-saving reduction in the amount of amino acid and reagents makes the methods of the invention suitable for large scale synthesis of T-20 and T-20-like peptides.

In addition, the inventors have surprisingly found that certain peptide fragments may be synthesized in the solid phase at a loading of about 0.8 to 1 mmol per gram of solid phase resin. This loading is significantly greater than the loading range of 0.25 to 0.4 mmol per gram of resin typically achieved in solid phase peptide synthesis. Moreover, the inventors have found that synthesizing selected peptide fragments in the solid phase using super acid sensitive resin produces peptide fragments of unusually high purity. Chromatographic techniques are not necessary to purify the peptide fragments produced according to the invention; the fragments are simply put through precipitation and/or trituration steps before use, or used as obtained directly from the resin. Use of a super acid sensitive resin allows the synthesized, protected peptides of the invention to be cleaved from the resin without concomitant removal of the side-chain protecting groups. This reduces impurities, and allows peptides comprising 10 amino acids or greater to be

synthesized in high purity. The impurity profile of T-20 and T-20-like peptides which are synthesized in the solution phase according to the methods of the invention by coupling of the high purity peptide fragments produced according to the invention consists of mainly fragments that did not couple, rather than closely related analogues. Accordingly, T-20 and T-20-like peptides produced according to the invention are much easier to purify than those produced according to conventional techniques. The Examples presented in Sections 9 and 11, below, demonstrate such combinatorial syntheses of T-20 full length peptides. The examples present in Section 11 demonstrates the large scale synthesis and purification of T-20 and T-20 intermediate peptides. Accordingly, the methods of the invention T-20 and T-20 peptide intermediates may be produced on a scale of one or more kilograms.

The present inventors have also unexpectedly found that peptides such as T-20 and other T-20-like peptides, as well as certain peptide fragments described herein may be purified using high capacity materials which can be used at basic pH ranges. Thus, the present invention still further relates to methods for the purification of peptides, in particular T-20 and T-20-like peptides, and the individual peptide fragments which act as intermediates in the synthesis of the subject peptides.

3.1 DEFINITIONS

The amino acid notations used herein are conventional and are as follows:

Common Amino Acid Abbreviations

Amino Acid	One-Letter Symbol	Common Abbreviation
Alanine	A	Ala
Asparagine	N	Asn
Aspartic acid	D	Asp

Amino Acid	One-Letter Symbol	Common Abbreviation
Glutamine	Q	Gln
Glutamic acid	E	Glu
Histidine	H	His
Isoleucine	I	Ile
Leucine	L	Leu
Lysine	K	Lys
Phenylalanine	F	Phe
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Tyrosine	Y	Tyr

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1: T-20 four fragment approach. This figure depicts the scheme followed in the Example presented in Section 9.1, below, for the synthesis of full-length T-20 beginning with intermediate peptide fragment Group 6, as shown in Table 2, above.

FIG. 2: T-20 four fragment approach, route 2. This figure depicts an additional four fragment scheme which couples peptide intermediate Group 6, as shown in Table 2, above, for the synthesis of full-length T-20.

FIG. 3: T-20 three fragment approach. This figure depicts the scheme followed in the Example presented in Section 9.1, below, for the synthesis of full-length T-20.

FIG. 4. T-20 three fragment approach, route 2. This figure depicts the scheme followed in the Example presented in Section 9.2, 9.3, 9.4 and 9.5, below, for the synthesis of full-length T-20.

FIG. 5. T-20 two fragment approach. This figure depicts a scheme which couples peptide intermediate Group 18 as shown in Table 2, above, for the synthesis of full-length T-20.

5. DETAILED DESCRIPTION OF THE INVENTION

5.1 FULL LENGTH PEPTIDES

The present invention relates to methods, peptide fragments, groups of peptide fragments which can be used to synthesize the peptide known as T-20, or alternatively, DP-178. T-20 is a peptide which corresponds to amino acid residues 638 to 673 of the transmembrane protein gp41 from HIV-1_{LAI} isolate and has the 36 amino acid sequence (reading from amino, NH_2, to carboxy, COOH, terminus):

NH_2-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-COOH

It will be understood that the methods, fragments and groups of fragments and techniques utilized for choosing the fragments and groups of fragments of the present invention may be used to synthesize T-20-like fragments in addition to T-20. The term "T-20-like" as used herein means any HIV or non-HIV peptide listed in U.S. Patent Nos. 5,464,933; 5,656,480 or PCT Publication No. WO 96/19495, each of which is hereby incorporated by reference in its entirety.

In addition to T-20 and the T-20-like peptides described above, the methods, fragments and groups of fragments of the present invention may be used to synthesize peptides having modified amino and/or carboxyl terminal ends. Taking T-20 as an example, such peptides can be of the formula:

X-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-Z

wherein X represents an amino group; a hydrophobic group selected from the group consisting of carbobenzoxyl, dansyl, and T-butyloxycarbonyl; an acetyl group; a 9-fluoroenyl-

methoxy-carbonyl (FMOC) group; or a macromolecular carrier group selected from the group consisting of lipid-fatty acid conjugates, polyethylene glycol, and carbohydrates; and Z represents a carboxyl group; an amido group; a T-butyloxycarbonyl group; a para-nitrobenzyl ester group; or a macromolecular carrier group selected from the group consisting of lipid-fatty acid conjugates, polyethylene glycol, and carbohydrates. Techniques for addition of such "X" and "Z" groups are well known to those of skill in the art.

In a preferred embodiment, the methods of the invention are used to synthesize the peptide having the above formula wherein X is an acetyl group and Z is an amide group. The Examples presented in Section 9, below, demonstrate the successful synthesis of T-20 peptides via coupling of peptide intermediates described, below, in Section 5.2. In a preferred method, T-20 and T-20-like peptides and intermediates can be purified using any non-silica based column packing (for maximization of loading capacity) including but not limited to zirconium-based packings, poly-styrene, poly-acrylic or other polymer based packings which are stable at high (greater than seven) pH ranges. For example, among the non-silica-laded column packing exhibiting a broad pH range that includes pH valves greater than that are sold by Tosohaus (Montgomeryville, PA). Columns packed with such material can be run in low, medium or high pressure chromatography. See, for example, the purification method presented in Section 10, below.

5.2 PEPTIDE INTERMEDIATES

The present invention encompasses, but is not limited to, peptide fragment intermediates of T-20 and T-20-like peptides with specific amino acid sequences as listed in Table 1 above, and the groups of peptide fragment intermediates listed in table 2. Such peptide intermediates,

especially in groups as listed in Table 2, below, can be utilized to produce T-20 and T-20 like peptides.

Any one or more of the side-chains of the amino acid residues of peptide fragments listed in Table 1 or 2 may be protected with standard protecting groups such as t-butyl (t-Bu), trityl (trt) and t-butyloxycarbonyl (Boc). The t-Bu group is the preferred side-chain protecting group for amino acid residues Tyr(Y), Thr(T), Ser(S) and Asp(D); the trt group is the preferred side-chain protecting group for amino acid residues His(H), Gln(Q) and Asn(N); and the Boc group is the preferred side-chain protecting group for amino acid residues Lys(K) and Trp(W).

During the synthesis of fragments 1, 2, 3 and 4 listed in Table 1, the side-chain of the histidine residue must be protected, preferably with a trityl (trt) protecting group. If it is not protected, the acid used to cleave the peptide fragment from the resin will detrimentally react with the histidine residue, causing degradation of the peptide fragment.

Preferably, the glutamine residues of the peptide fragments of the invention are protected with trityl (trt) groups. However, it is preferred not to protect the glutamine residue at the carboxy-terminal end of fragments 1-16 and 9-16. It has been found that the absence of a protective group on the glutamine residue at the carboxy-terminal end of the 1-16 fragment facilitates reaction of the 1-16 fragment with the 17-36 fragment, allowing coupling of the fragments with only about 2% racemization. In addition, if lower solubility of any of the peptide fragments of the invention in organic solvents is desired, the trityl protecting groups may be eliminated from any one or more of the other glutamine residues of the fragments.

Preferably, all the asparagine residues of each peptide fragment of the invention are protected. In addition, it is

preferred that the tryptophan residue is protected with a Boc group.

Protected peptide fragments according to peptide formulas 1-18 listed in Table 1 above include, but are not limited to, the compounds listed in Table 3 below.

TABLE 3

Peptide Formula No.	Formula	Corresponding Numbered Amino Acid Sequence of T-20
1a	Ac-YTSLIHSL-COOH (SEQ ID NO:2)	1-8
1b	FMOC-YTSLIHSL-COOH (SEQ ID NO:2)	1-8
3a	Ac-YTSLIHSLIEESQNQQ-OPNB(SEQ ID NO:4)	1-16
3b	FMOC-YTSLIHSLIEESQNQQ-OPNB (SEQ ID NO:4)	1-16
3c	Ac-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4)	1-16
3d	FMOC-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4)	1-16
5a	Ac-IEESQNQ-COOH (SEQ ID NO:6)	9-15
5b	FMOC-IEESQNQ-COOH (SEQ ID NO;6)	9-15
6a	NH_2-IEESQNQQ-OPNB (SEQ ID NO:7)	9-16
6b	FMOC-IEESQNQQ-OPNB (SEQ ID NO:7)	9-16
9a	Ac-EKNEQEL-COOH (SEQ ID NO:10)	17-23
9b	FMOC-EKNEQEL-COOH (SEQ ID NO:10)	17-23
10a	Ac-EKNEQELLEL-COOH (SEQ ID NO:11)	17-26
10b	FMOC-EKNEQELLEL-COOH (SEQ ID NO:11)	17-26

Peptide Formula No.	Formula	Corresponding Numbered Amino Acid Sequence of T-20
11b	NH_2-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)	17-36
11b	FMOC-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)	17-36
11b	Ac-LELDKWASLWNW-COOH (SEQ ID NO:15)	24-35
14b	FMOC-LELDKWASLWNW-COOH (SEQ ID NO:15)	24-35
15a	NH_2-LELDKWASLWNWF-NH_2 (SEQ ID NO:16)	24-36
15b	FMOC-LELDKWASLWNWF-NH_2 (SEQ ID NO:16)	17-36
16a	Ac-DKWASLWNW-COOH (SEQ ID NO:17)	27-35
16b	FMOC-DKWASLWNW-COOH (SEQ ID NO:17)	27-35
17a	NH_2-DKWASLWNWF-NH_2 (SEQ ID NO:18)	27-36
17b	FMOC-DKWASLWNWF-NH_2 (SEQ ID NO:18)	27-36
18A	FMOC-EKNEQELLELDKWASLWNW-COOH (SEQ ID NO:19)	17-35

Any one or more of the side-chains of the amino acid residues of the peptides listed in Table 3 above may be protected with standard side-chain protecting groups such as tBu, trt and Boc, as described above. Representative synthesis of peptides from Table 3 are presented in Sections 7 and 8, below, which utilize the general techniques discussed in Section 5.4, below.

5.3 PEPTIDE SYNTHESIS

As discussed above, some of the individual peptide fragments of the invention are preferably made using solid phase synthesis techniques, while other peptides of the invention are preferably made using a combination of solid phase and solution phase synthesis techniques, said syntheses culminating in the production of T-20 or T-20-like peptides as described herein. However, it will be understood that the peptide fragments of the invention may be synthesized or prepared by techniques well known in the art. See, for example, Creighton, 1983, Proteins: Structures and Molecular Principles, W.H. Freeman and Co., NY, which is incorporated herein by reference in its entirety.

The peptides of the invention may alternatively be synthesized such that one or more of the bonds which link the amino acid residues of the peptides are non-peptide bonds. These alternative non-peptide bonds may be formed by utilizing reactions well known to those in the art, and may include, but are not limited to imino, ester, hydrazide, semicarbazide, and azo bonds, to name but a few.

In yet another embodiment of the invention, T-20 and T-20 like peptides comprising the sequences described above may be synthesized with additional chemical groups present at their amino and/or carboxy termini, such that, for example, the stability, reactivity and/or solubility of the peptides is enhanced. For example, hydrophobic groups such as carbobenzoxyl, dansyl, acetyl or t-butyloxycarbonyl groups, may be added to the peptides' amino termini. Likewise, an acetyl group or a 9-fluorenylmethoxy-carbonyl group may be placed at the peptides' amino termini. (See "X" modification of T-20, described above.) Additionally, the hydrophobic group, t-butyloxycarbonyl, or an amido group may be added to the peptides' carboxy termini. Similarly, a para-nitrobenzyl ester group may be placed at the peptides' carboxy termini. (See "Z" modification of T-20, described above.) Techniques

for introducing such modifications are well known to those of skill in the art.

Further, T-20 and T-20-like peptides may be synthesized such that their steric configuration is altered. For example, the D-isomer of one or more of the amino acid residues of the peptide may be used, rather than the usual L-isomer.

Still further, at least one of the amino acid residues of the peptides of the invention may be substituted by one of the well known non-naturally occurring amino acid residues. Alterations such as these may serve to increase the stability, reactivity and/or solubility of the peptides of the invention.

Any of the T-20 or T-20-like peptides may be synthesized to additionally have a macromolecular carrier group covalently attached to their amino and/or carboxy termini. Such macromolecular carrier groups may include, for example, lipid-fatty acid conjugates, polyethylene glycol, carbohydrates or additional peptides. The "X" modification of T-20 described above may therefore additionally represent any of the above macromolecular carrier groups covalently attached to the amino terminus of a peptide, with an additional peptide group being preferred. Likewise, the "Z" modification of T-20 described above may additionally represent any of the macromolecular carrier groups described above.

Preferably, the peptide fragments of the present invention are synthesized by solid phase peptide synthesis (SPPS) techniques using standard FMOC protocols. See, e.g., Carpino et al., 1970, J. Am. Chem. Soc. 92(19):5748-5749; Carpino et al., 1972, J. Org. Chem. 37(22):3404-3409. In a preferred embodiment, the solid phase synthesis of the peptide fragments of the present invention is carried out on super acid sensitive solid supports which include, but are not limited to, 2-chlorotrityl chloride resin (see, e.g., Barlos et al., 1989, Tetrahedron Letters 30(30):3943-3946)

and 4-hydroxymethyl-3-methoxyphenoxybutyric acid resin (see, e.g., Seiber, 1987, Tetrahedron Letters 28(49):6147-6150, and Richter et al., 1994, Tetrahedron Letters 35(27):4705-4706). Both the 2-chlorotrityl chloride and 4-hydroxymethyl-3-methoxyphenoxy butyric acid resins may be purchased from Calbiochem-Novabiochem Corp., San Diego, CA.

General procedures for production and loading of resins which can be utilized in solid phase peptide synthesis are described herein. Resin loading can be performed, for example, via the following techniques: The resin, preferably a super acid sensitive resin such as 2-chlorotrityl resin, is charged to the reaction chamber. The resin is washed with a chlorinated solvent such as dichloromethane (DCM). The bed is drained and a solution of 1.5 equivalents of an amino acid and 2.7 equivalents of diisopropylethylamine (DIEA) in about 8-10 volumes of dichloroethane (DCE) is added. The N-terminus of the amino acid should be protected, preferably with Fmoc, and the side chain of the amino acid should be protected where necessary or appropriate. The mixture is agitated with nitrogen bubbling for 2 hours.

It should be noted that a chlorinated solvent is required for adequate swelling of the 2-chlorotrityl resin. Although DCE provides greater loading efficiency according to literature sources, DCM may be substituted with little or no reduction in the loading.

After agitation, the bed is drained and washed with DCM. The active sites on the resin are endcapped with a 9:1 MeOH:DIEA solution for about 20-30 minutes. The bed is drained, washed 4x with DCM and dried with a nitrogen purge to give the loaded resin.

Fmoc is the preferred protecting group for the N-terminus of the amino acid. Depending on which amino acid is being loaded, its side chain may or may not be protected. For example, when Trp is loaded, its side chain should be protected with Boc. Similarly, the side-chain of Gln may be protected with trt. However, when Gln is being loaded in

preparation for the synthesis of the 1-16 peptide fragment, its side chain should not be protected. It is not necessary to protect the side-chain of Leu.

The Fmoc-protected amino acids used in loading the resin and in peptide synthesis are available, with or without side-chain protecting groups as required, from Sean or Genzyme. As an alternative to the above procedure, the resin may be purchased already loaded with the appropriate amino acid.

The Examples presented in Section 6, below, describe exemplary resin preparations.

Solid phase peptide synthesis techniques can be performed as, for example, according to the following techniques: The loaded resin is added to the reaction chamber and conditioned with a solvent, preferably methylene chloride (DCM; at preferably about 10 vol.) with nitrogen agitation for about 15 minutes to swell the resin beads. DCM is required for adequate swelling of the 2-chlorotrityl resin. The resin volume will double or triple in the reaction chamber as the beads swell and the active sites unfold and become accessible to reaction. After the resin is swelled, the solvent is drained from the reaction chamber.

Removal of the Fmoc (9-fluroenyl-methyloxycarbonyl) protecting group from the terminal amine or the resin is accomplished by treating the resin with 2 aliquots of a 20% solution of piperidine in N-methyl-2-pyrrolidinone (NMP) for about ten minutes each. The volume of the 20% solution of piperidine in NMP required for each aliquot will depend on the scale of the reaction being run. The resin is then washed 5-7 times with aliquots of NMP (about10 vol.) to remove the Fmoc by-products (i.e., dibenzofulvene and its piperidine adduct) and residual piperidine.

A chloranil test may be used to determine if the removal of Fmoc by-products and residual pyridine is complete. The chloranil test solution is prepared by adding a drop of a saturated solution of chloranil in toluene to about 1 mL of acetone. The NMP washings may be tested by adding a drop of

the washing to the chloranil test solution. A blue or violet color is a positive indication for the presence of secondary amine, indicating that Fmoc by-products and/or residual piperidine are still present. The NMP washing is repeated until the blue or violet color is no longer observed.

Meanwhile, the subsequent amino acid in the sequence to be added to the resin is activated for reaction at its carboxy terminus. The amine terminus of each amino acid should be protected with Fmoc. Depending on which amino acid is being added, its side chain may or may not be protected. Preferably, the side-chains of tyr(Y), Thr(T), Ser(S) and Asp(P) are protected with t-Bu, the side-chains of His(H), Gln(Q) and Asn(N) are protected with trt, and the side-chains of Lys(K) and Trp(w) are protected with Boc. However, as discussed above, the side-chain of His must be protected. Moreover, it is preferred <u>not</u> to protect the side-chain of the Gln residue at the carboxy-terminal end of fragments 1-16 and 9-16. It is not necessary for the side-chains of Len or Ile to be protected.

The amino acid is activated as follows. The Fmoc-protected amino acid (1.5 eq), 1-hydroxybenzotriazole hydrate (HOBT) (1.5 eq), and diisopropyl-ethylamine (DIEA) (1.5 eq) are dissolved in NMP (about 7.5 vol.) at room temperature. The solution is chilled to 0-5°C, and then O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) (1.5 eq) is added followed by stirring for 5-15 minutes to dissolve. It is important that activation is carried out at low temperature to minimize racemization of the amino acid. The HBTU is the last reagent added to the cold solution since activation and racemization cannot take place in its absence.

The solution of activated amino acid is charged to the drained resin, washing in with DCM (approximately 2.5 vol). Note that activation of the amino acid is carried out in NMP due to the insolubility of HBTU in DCM. However, DCM is added to the reaction at this point to maintain adequate swelling of the resin beads. The reaction is agitated with N_2

bubbling for about 1 hour. Coupling completion may be monitored with a qualitative ninhydrin test as described below.

To check for completion of the reaction using the qualitative ninhydrin test, a 2-20 mg sample of the resin is withdrawn and washed clean with methanol. To the sample is added 3 drops of a 76% solution of phenol in ethanol, 4 or 5 drops of a 0.2 mM KCN solution in pyridine, and 3 drops of a 0.28 M solution of ninhydrin in ethanol. The sample is diluted with ethanol to a volume of about 0.5 mL and placed in a heat block at about 75°C for 5-10 minutes. A blue or violet color is a positive indication for the presence of free amines, indicating that the reaction is not yet complete. The sample can be diluted further to a volume of about 3 mL to more easily gauge the degree of color change in the concentrated sample.

If a positive ninhydrin test is observed after one hour, the coupling reaction is continued for an additional hour. If the positive ninhydrin test persists after 2 hours, the resin is drained, washed three times in approximately 10 volumes of NMP, and the coupling reaction is repeated using 1 equivalent of activated amino acid.

If the resin is to be stored overnight between coupling cycles, the resin bed may be drained and covered with DCM under a nitrogen blanket. Alternatively, the bed may be drained, stored under a nitrogen blanket, then conditioned with a DCM wash prior to proceeding with the next coupling cycle. If the completed fragment is to be stored overnight prior to cleavage, the resin bed should be washed free of NMP with DCM because significant Fmoc deprotection can occur in NMP.

After the coupling is judged complete, the resin is drained and washed with 3 aliquots (approximately 10 vol.) of NMP. The cycle is repeated for subsequent mers of the peptide fragment. Following the final coupling reaction, the resin is washed with 4 aliquots (about 10 vol.) of NMP, then

with 4 aliquots (approximately 10 vol.) of DCM. The resin-bound peptide may be dried with a nitrogen purge.

Peptides synthesized via solid phase synthesis techniques can be cleaved and isolated according to, for example, the following techniques: The peptide may be cleaved from the resin using techniques well known to those skilled in the art. For example, solutions of 1% or 2% trifluoroacetic acid (TFA) in DCM or a combination of a 1% and a 2% solution of TFA in DCM may be used to cleave the peptide. Acetic acid (HOAC) may also be used to cleave the peptide. The specific cleavage reagent, solvents and time required for cleavage will depend on the particular peptide being cleaved. After cleavage the cleavage fractions are subjected to standard work-up procedures to isolate the peptide. Typically, the combined cleavage fractions are concentrated under vacuum, followed by reconstitution with a solvent such as ethanol, methanol or heptane. In general, the peptide is precipitated by the addition of water, and collected by vacuum filtration. Alternatively, the product may be triturated prior to isolation of the peptide.

The Examples presented in Sections 7.1 - 7.6, below, present solid phase syntheses of peptide intermediates as shown in Tables 1, 2 and/or 3.

For synthesis of full length T-20 peptides, the peptide intermediates of Table 1, above, can be coupled together to yield the T-20 peptide. For example, the groups of peptide intermediates listed in Table 2, above, can be coupled together to produce T-20 full-length peptide. Representative examples of such synthesis of full-length T-20 from intermediate peptide fragments are presented in Section 9, below, and are d epicted schematically in FIGS. 1-5.

In certain embodiments, a four fragment approach for synthesis of T-20 can be followed. A "four fragment approach" synthesis refers to a T-20 synthesis scheme which begins with four T-20 intermediate peptide fragments that are synthesized and coupled using solid and liquid phase

synthesis techniques into a full-length T-20 peptide. Intermediate peptide fragment groups 5, 6, 8, 9 and 12-15 shown in Table 2, above, represent preferred groups. FIGS. 1 and 2 depict two four fragment approaches which utilize Table 2 peptide intermediate Group 6 to synthesize full-length T-20. For this group, it is noted that amino acid residue 36 (the T-20 carboxy l-terminal amino acid residue) is introduced individually during the fragment coupling process. The culmination of the T-20 synthesis scheme shown in FIG. 1 is demonstrated in the example presented in Section 9.1.

In addition, embodiments, a three fragment approach for synthesis of T-20 can be followed. A "three fragment approach" synthesis refers to a T-20 synthesis scheme which begins with three T-20 intermediate peptide fragments that are synthesized and coupled using solid and liquid phase synthesis techniques into a full-length T-20 peptide. Intermediate fragment groups 2-4, 7, 10 and 11 shown in Table 2, above, represent preferred three fragment groups. FIGS. 3 and 4 depict two three fragment approaches which utilize Table 2 peptide intermediate Group 3 to synthesize full-length T-20. For this group, it is noted that amino acid residue 36 (the T-20 carboxyl-terminal amino acid residue) is introduced individually during the fragment coupling process. The culmination of the T-20 synthesis scheme shown in FIG. 3 is demonstrated in the example presented in Section 9.1, below. The culmination of the T-20 synthesis scheme shown in FIG. 4 is demonstrated in the examples presented in Sections 9.2-9.5, below.

In additional embodiments, a two fragment approach for synthesis of T-20 can be followed. A "two fragment approach" synthesis refers to a T-20 synthesis scheme which begins with two T-20 intermediate peptide fragments that are synthesized and coupled using solid and liquid phase synthesis techniques into a full-length T-20 peptide. Intermediate fragment Groups 1 and 16-20 shown in Table 2, above, represent preferred to fragment groups. FIG. 5 depicts a two fragment

approach which utilizes Table 2 peptide intermediate Group 20 to synthesize full-length T-20. For this group, it is noted that amino acid residue (the T-20 carboxyl-terminal amino acid residue) is introduced individually during the fragment coupling process.

Solution phase peptide synthesis techniques well known to those of skill in the art may be utilized for synthesis of the peptide intermediate fragments of the invention. The Examples presented in Sections 8.1 - 8.11 describe exemplary solution phase peptide syntheses of peptide intermediates listed in Tables 1, 2 and/or 3. For example, among the non-silica-laded column packing exhibiting a broad pH range that includes pH valves greater than that are sold by Tosohaus (Montgomeryville, PA).

6. EXAMPLE: Resin Syntheses

Described herein, in Sections 6.1-6.3, are examples in which chlorotrityl resins are synthesized which can be utilized in conjunction with solid phase synthesis of the peptides and peptide intermediates described herein.

6.1 Preparation of Fmoc-Trp(Boc)-2-Chlorotrityl Resin

Materials:	MW	eq	mmoles	grams	mL
2-Chlorotritylchloride resin	--	1.0	25	25	--
Fmoc-Trp(Boc)-OH	--	526.60	1.5	37.5	19.7
Diisopropylethyl amine (DIEA)	129.25	1.7	42.5	5.5	7.4
Dichloroethane (DCE)	--	--	--	--	250
Dichloromethane (DCM)	--	--	--	--	6 X 250
9:1 Methanol:DIEA	--	--	--	--	200

Procedure:

The 2-chlorotrityl chloride resin (25 g, 1 eq.) was charged to a 500 mL peptide chamber and washed with 250 mL of DCM. The bed was drained and a solution of the Fmoc-Trp(Boc)-OH (1.5 eq) and the DIEA (1.7 eq) in 10 volumes of DCE was added. The mixture was agitated with nitrogen bubbling for 2 hrs.

The bed was drained and washed with 250 mL DCM. The active sites on the resin were end-capped with 200 mL of a

9:1 MeOH:DIEA solution for 20 minutes. The bed was drained, washed with 4 x 250 mL of DCM, and dried with a nitrogen purge to give 34.3 g of loaded resin.

Quantitative HPLC analysis was performed by cleaving the Fmoc-amino acid from the resin and assaying versus a standard. HPLC assay of the material showed a loading of the resin at 0.68 mmol/g.

Column: Phenomenox Jupiter C18; 300Å; 5μ
Flow rate: 1 mL/min
Detection: UV at 260 nm

Mobile phase: A: 0.1% aqueous TFA
B: 0.1% TFA in acetonitrile
65% B isocratic

Retention time: ~ 14 minutes

6.2 Preparation of Fmoc-Gln-2-Chlorotrityl Resin

Materials:	MW	eq	mmoles	grams	mL
2-Chlorotritylchloride resin	--	1.0	25	25	--
FmocGlnOH	368.39	1.5	37.5	13.8	--
Diisopropylethyl amine (DIEA)	129.25	1.7	42.5	5.5	7.4
Dichloroethane (DCE)	--	--	--	--	75
N,N-Dimethylformamide (DMF)	--	--	--	--	200
Dichloromethane (DCM)	--	--	--	--	6 X 250
9:1 Methanol:DIEA	--	--	--	--	200

Procedure:

The procedure used in the Example presented in 6.1, above, was repeated using a solution of FmocGlnOH (1.5 eq) and DIEA (1.7 eq.) in a mixture of 75 mL DCE and 200 mL DMF. The addition of DMF to stabilizes the FmocGlnOH. The reaction yielded 33.8 g of loaded resin. A theoretical loading of the resin at 0.74 mmol/g was assumed and the material was carried forward.

6.3 Preparation of Fmoc-Leu-2-Chlorotrityl Resin

Materials:	MW	eq	mmoles	grams	mL
2-Chlorotritylchloride resin	--	1.0	250	250	--
FmocLeuOH	353.42	1.5	375	132.5	--
Diisopropylethyl amine (DIEA)	129.25	1.7	425	55	75
Dichloroethane (DCE)	--	--	--	--	2000
Dichloromethane (DCM)	--	--	--	--	6 X 1500
9:1 Methanol:DIEA	--	--	--	--	1500

Procedure:

The resin was charged to a 3 L peptide chamber and washed with 1.5 DCM. The bed was drained and a solution of the FmocLeuOH (1.5 eq) and the DIEA (1.7 eq) in 8 volumes of DCE was added. The mixture was agitated with nitrogen bubbling for 2 hrs.

The bed was drained and washed with 1.5 L DCM. The active sites on the resin were end-capped with 1.5 L of a 9:1 MeOH:DIEA solution for 30 minutes. The bed was drained, washed with 4 x 1.5 L of DCM, and dried with a nitrogen purge to give 345 g of loaded resin.

Quantitative HPLC analysis was performed by cleaving the Fmoc-amino acid from the resin and assaying versus a standard. HPLC assay of the material showed a loading of the resin at 0.72 mmol/g.

Column: Phenomenox Jupiter C18; 300Å; 5μ
Flow rate: 1 mL/min
Detection: UV at 260 nm
Mobile phase: A: 0.1% aqueous TFA
B: 0.1% TFA in acetonitrile
65% B isocratic

Retention time: ~ 8 minutes

7. EXAMPLE: SOLID PHASE SYNTHESIS OF PEPTIDES

Presented below, in Sections 7.1-7.6, are examples of the solid phase synthesis of peptide intermediates as listed in Tables 1, 2, and/or 3.

7.1 Preparation of Fragment Fmoc-AA(1-8)-OH (Fragment 1b)

Structure:

Fmoc-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-OH (SEQ ID NO:2)

$C_{93}H_{121}N_{10}O_{15}$
MW 1619.06

Materials:	MW	eq	mmoles	grams	mL
Fmoc-Leu-2-chlorotrityl resin	--	1.0	15.6	20.0	--
Fmoc-amino acid*	--	1.5	23.4	--	--
1-Hydroxybenzotriazole(HOBT) hydrate*	153.15	1.5	23.4	3.6	--
O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate(HBTU)*	379.25	1.5	23.4	8.9	--
Diisopropylethylamine(DIEA)*	129.25	1.5	23.4	3.0	4.1
N-methyl-2-pyrrolidinone(NMP)*	--	--	--	--	150
Methylene chloride(DCM)*	--	--	--	--	50
20% piperidine/NMP*	--	--	--	--	2 x 200
NMP for rinsing*	--	--	--	--	200(per wash)
1% Trifluoroacetic acid(TFA) in DCM	--	--	--	--	300
0.5% TFA/DCM	--	--	--	--	200
Pyridine	--	--	--	--	
Ethyl alcohol	--	--	--	--	110
Water	--	--	--	--	200 + 100

*per coupling cycle

Theoretical Yield: 25.3 g

Expected Yield: 80-90%
Actual Yield: 20.0g

Procedure:

To a 1L peptide reaction chamber was charged 20.0g Fmoc-Leu-2-chlorotrityl resin. The resin was conditioned in 200 mL (~10 vol) of DCM with nitrogen agitation for about 15 minutes to swell the beads, then drained.

Fmoc (9-fluorenylmethyloxycarbonyl) removal from the terminal amine was accomplished using 2 X 200 mL of a 20% solution of piperidine in NMP for 10 minutes each. The resin was then washed 5-7 times with 200 mL (~10 vol) of NMP to remove Fmoc by-products (dibenzofulvene and its piperidine adduct) and residual piperidine, as determined by a negative chloranil test.

Meanwhile, Fmoc-Ser (tBu), the subsequent amino acid in the sequence was activated for reaction at the carboxyl

terminus. The Fmoc-protected amino acid (1.5 eq), the HOBT (1.5 eq), and the DIEA (1.5 eq) were dissolved in 150 mL (~7.5 vol) of NMP at room temperature. The solution was chilled to 0-5°C, then the HBTU (1.5 eq) was added and stirred 5-15 minutes to dissolve. The solution of activated acid was charged to the drained resin, washing in with 50 mL of DCM (~2.5 vol). The reaction was agitated with N_2 bubbling for 1 hr. Coupling completion was monitored with the qualitative ninhydrin test. After the coupling reaction was judged complete, the resin was drained and washed 3 X 200 mL (1 vol) of NMP.

The cycle was repeated for subsequent mers of the peptide fragment using 1.5 equivalents each of Fmoc-protected amino acids His(trt), Ile, Leu, Ser(tBu), Thr(tBu) and Tyr(tBu). Following the final coupling reaction, the resin was washed 4 X 200 mL (10 vol) of NMP, then with 4 X 200 mL (10 vol) of DCM. The resin was dried with a nitrogen purge to give 42 g of resin-bound peptide.

The peptide was cleaved from a 21 g quantity of the resin using 300 mL of 1% TFA in DCM for about 2 minutes, followed by 200 mL of 0.5% TFA in DCM. The cleavage fractions were collected onto pyridine (1:1 volume ratio to TFA). The cleavage washes were combined and concentrated under vacuum to a volume of ~50 mL, then reconstituted with 110 mL of ethanol while the concentration was continued to remove residual DCM to a final volume of ~250 mL. Product was precipitated with the addition of 200 mL of water. The slurry was stirred at room temperature for 30 minutes. The solids were collected by vacuum filtration and washed with ~100 mL of water. The product was air dried to give 20.0 g (79%) of Fmoc-AA(1-8)-OH of 95% HPLC purity.

Column: Phenomenox Jupiter C18
Flow rate: 1 mL/min
Detection: UV at 260 nm

Mobile phase: A: 0.1% aqueous TFA
B: 0.1% TFA in acetonitrile gradient from 80% B to 99% B in 20 minutes

Retention time: about 23 minutes

7.2 Preparation of Fragment Fmoc-AA(9-15)-OH (Fragment 5b) Structure:

Fmoc-Ile-Glu(tBu)-Glu(tBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-OH (SEQ ID NO:6)

$C_{117}H_{129}N_{10}O_{18}$
MW 1963.39

Materials:	MW	eq	mmoles	grams	mL
Fmoc-Gln(trt)-2-chlorotrityl resin	--	1.0	12.0	20.0	--
Fmoc-amino acid*	--	1.5	18.0	--	--
1-Hydroxybenzotriazole(HOBT) hydrate*	153.15	1.5	18.0	2.8	--
O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate(HBTU)*	379.25	1.5	18.0	6.8	--
Diisopropylethylamine(DIEA)*	129.25	1.5	18.0	2.3	3.1
N-methyl-2-pyrrolidinone(NMP)*	--	--	--	--	150
Methylene chloride(DCM)*	--	--	--	--	50
20% piperidine/NMP*	--	--	--	--	2 x 200
NMP for rinsing*	--	--	--	--	200(per wash)
DCM for cleavage	--	--	--	--	160
Acetic acid (HOAc)	--	--	--	--	20
Trifluoroethanol	--	--	--	--	20
Heptane	--	--	--	--	250+250+100
Methyl t-butyl ether	--	--	--	--	100
Isopropanol	--	--	--	--	60
Water	--	--	--	--	60 + 50

*per coupling cycle

Theoretical Yield: 23.6 g

Expected Yield: 89-95%
Actual Yield: 21.1g

Procedure:

The procedure used in the Example presented in Section 7.1, above was repeated using 20.0g Fmoc-Gln(trt)-2-chlorotrityl resin, and Fmoc-protected amino acids Asn(trt), Gln(trt), Ser(tBu), Glu(tBu), Glu(tBu) and Ile.

Following the final coupling reaction, the resin was washed 4 X 200 mL (10 vol) of NMP, then with 4 X 200 mL (10 vol) of DCM.

The peptide was cleaved from the resin using 200 mL of 8:1:1 DCM:TFE:HOAc for 2 hours, followed by 2 X 100 mL washes of DCM. The combined eluants were concentrated under vacuum to a volume of ~100 mL, then reconstituted with 250 mL of heptane while the concentration was continued to remove residual DCM to a final volume of ~250 mL. The heptane layer was separated from the biphasic mixture which formed. The product was precipitated with the addition of 250 mL of heptane and 100 mL of MTBE, then triturated overnight at room temperature to give material of desired consistency. The solids were collected by vacuum filtration and washed with about 100 mL of heptane. The product was reworked to remove residual acetic acid. The filtered solids were dissolved in 60 mL of isopropanol at 50°C. The solution was chilled in an ice bath to 0-5°C, then 60 mL of water was added at a rapid dropwise rate. The product slurry was triturated with stirring for ~1 hour in the ice bath. The solids were isolated by vacuum filtration and washed with ~50 mL of water. The product was air dried to give 21.1 g (90%) of Fmoc-AA(9-15)-OH of 95% HPLC purity.

Column: Phenomenox Jupiter C18
Flow rate: 1 mL/min
Detection: UV at 260 nm
Mobile phase: A: 0.1% aqueous TFA
B: 0.1% TFA in acetonitrile gradient from 80% B to 99% B in 20 minutes

Retention time: ~23 minutes

7.3 Preparation of Fragment Fmoc-AA(1-16)-OH (Fragment 3d)

Structure:

Fmoc-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(tBu)-Glu(tBu)-Ser(tBu)Gln(trt)-Asn(trt)-Gln(trt)-Gln-OH (SEQ ID NO:4)

$C_{199}H_{245}N_{22}O_{32}$
MW 3457.30

Materials:	MW	eq	mmoles	grams	mL
Fmoc-Gln-2-chlorotrityl resin	--	1.0	24.1	32.5	--
Fmoc-amino acid*	--	1.5	36.2	--	--
1-Hydroxybenzotriazole(HOBT) hydrate*	153.15	1.5	36.2	5.5	--
O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate(HBTU)*	379.25	1.5	36.2	13.7	--
Diisopropylethylamine(DIEA)*	129.25	1.5	36.2	4.7	6.3
N-methyl-2-pyrrolidinone(NMP)*	--	--	--	--	200
Methylene chloride(DCM)*	--	--	--	--	75
20% piperidine/NMP*	--	--	--	--	2 x 250
NMP for rinsing*	--	--	--	--	250(per wash)
1% Trifluoroacetic acid/DCM	--	--	--	--	4 x 50
Pyridine	79.10	--	--	--	4 x 0.5
Heptane	--	--	--	--	150 + 50
Methanol	--	--	--	--	50
Water	--	--	--	--	50 + 25

*per coupling cycle

Theoretical Yield: 48.9 g **Expected Yield:** 85-90%

Procedure:

The procedure used in the Example presented in Section 7.1, above, was repeated using 32.5g Fmoc-Gln-2-chlorotrityl resin and the required Fmoc-protected amino acids. The reaction was run as described in the Example presented in Section 6.1, above, except slightly different volumes of solvents were used as indicated in the Materials section above.

Following the final coupling reaction, the resin was washed 4 X 250 mL (8 vol) of NMP, then with 4 X 250 mL (8 vol) of DCM. The resin was dried under a nitrogen purge to give 97.4 g of bound peptide.

On 17.7-g scale, the resin-bound peptide was cleaved from the resin using 2 X 190 mL of 1% TFA in DCM for 1-2

minutes, followed by 1 X 120 mL with DCM. The cleavage fractions were collected onto pyridine (1:1 volume ratio to TFA). The fractions and wash were combined and concentrated under vacuum to a volume of ~50 mL, then reconstituted with 200 mL of methanol. The concentration was continued to remove residual DCM to a final volume of ~50 mL. The product was precipitated with the addition of 250 mL of water and stirred at room temperature for ~30 minutes. The solids were collected by vacuum filtration and washed with ~50 mL of water. The product was air dried to give 12.8 g (84%). The product was reworked to remove pyridinium salts. The filtered solids were dissolved in 150 mL of methanol at room temperature. Addition of 200 mL of water at room temperature precipitated the product. The product was isolated by vacuum filtration and washed with about 50 mL of water. The material was air dried to give 12.8 g (84%) of Fmoc-AA(1-16)-OH.

Column: Phenomenox Jupiter C18
Flow rate: 1 mL/min
Detection: UV at 260 nm

Mobile phase: A: 0.1 % aqueous TFA
B: 0.1 % TFA in acetonitrile gradient from 75% B to 99% B in 20 minutes

Retention time: ~25 minutes

7.4 Preparation of Fragment Ac-AA(1-16)-OH (Fragment 3c)

Structure:

Ac-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(tBu)-Glu(tBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-OH (SEQ ID NO:4)

$C_{186}H_{237}N_{22}O_{31}$
MW 3274.76

Materials:	MW	eq	mmoles	grams	mL
Fmoc-Gln-2-chlorotrityl resin	--	1.0	24.1	32.5	--
Fmoc-amino acid*	--	1.5	36.2	--	--
1-Hydroxybenzotriazole(HOBT) hydrate*	153.15	1.5	36.2	5.5	--
O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate(HBTU)*	379.25	1.5	36.2	13.7	--
Diisopropylethylamine(DIEA)*	129.25	1.5	36.2	4.7	6.3
N-methyl-2-pyrrolidinone(NMP)*	--	--	--	--	200
Methylene chloride(DCM)*	--	--	--	--	75
20% piperidine/NMP*	--	--	--	--	2 x 250
NMP for rinsing*	--	--	--	--	250(per wash)
1% Trifluoroacetic acid/DCM	--	--	--	--	4 x 50
Pyridine	79.10	--	--	--	4 x 0.5
Heptane	--	--	--	--	150 + 50
Methanol	--	--	--	--	50
Water	--	--	--	--	50 + 25

*per coupling cycle

Theoretical Yield: 48.9 g **Expected Yield:** 85-90%

Procedure:

The procedure used in the Example presented in Section 7.1, above, was repeated using 32.5g Fmoc-Gln-2-chlorotrityl resin and the required Fmoc-protected amino acids, and the solvent volumes indicated in the materials section above.

Following the final coupling reaction, the resin was washed 4 X 250 mL (8 vol) of NMP, then with 4 X 250 mL (8 vol) of DCM. The resin was dried under a nitrogen purge to give 97.4 g of bound peptide.

On 10-g scale, the resin-bound peptide was acetylated with acetic anhydride and pyridine (5 eq each) in 100 mL of 3:1 NMP:DCM for 30 minutes, then washed with 2 X 25 mL DCM. The peptide was cleaved from the resin using 3 X 50 mL of 1 % TFA in DCM, followed by 2 X 50 mL washes of DCM. The cleavage fractions were collected onto pyridine (1:1 volume ratio to TFA). The fractions and wash were combined and concentrated under vacuum to a volume of about 100 mL, then reconstituted with 3 X 50 mL of heptane added portionwise while the concentration was continued to remove residual DCM to a final volume of ~150 mL. The product precipitated initially with a somewhat tacky consistency but triturated

with stirring for ~ 30 minutes in an ice bath at 0-5° to a filterable solid. The solids were collected by vacuum filtration and washed with ~ 50 mL of heptane. The product was reworked to remove pyridinium salts. The filtered solids were dissolved in 50 mL of methanol at room temperature. The solution was chilled in an ice bath to 0-5°C, then 50 mL of water was added at a rapid dropwise rate. The material initially precipitated as a tacky solid which triturated to filterable consistency with stirring for ~1 hour in the ice bath. The product was isolated by vacuum filtration and washed with ~ 25 mL of water. The product was air dried to give 7.0 g (90%) of AcAA(1-16)-OH. The product was subsequently reworked as described above to give 6.2g (89% recovery) of material of 96% HPLC purity.

Column: Zorbax LP C8, 100Å, 20μ
Flow rate: 1 mL/min
Detection: UV at 220 nm

Mobile phase: A: 0.1 % aqueous TFA
B: 1:1 ACN:IPA with 0.05%TFA gradient from 80% B to 99% B in 20 minutes

Retention time: ~15 minutes

7.5 Preparation of Fragment Fmoc-AA(17-26)-OH (Fragment 10b)

Structure:

Fmoc-Glu(tBu)-Lys(Boc)-Asn(trt)-Glu(tBu)-Gln(trt)-Glu(tBu)-Leu-Leu-Glu(tBu)-Leu-OH (SEQ ID NO:11)

$C_{127}N_{167}N_{13}O_{25}$
MW 2275.82

Materials:	MW	eq	mmoles	grams	mL
Fmoc-Leu-2-chlorotrityl resin	--	1.0	19.5	25.0	--
Fmoc-amino acid*	--	1.5	30.0	--	--
1-Hydroxybenzotriazole(HOBT) hydrate*	153.15	1.5	30.0	4.6	--
O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate(HBTU)*	379.25	1.5	30.0	11.4	--
Diisopropylethylamine(DIEA)*	129.25	1.5	30.0	3.9	5.2
N-methyl-2-pyrrolidinone(NMP)*	--	--	--	--	200
Methylene chloride(DCM)*	--	--	--	--	75

20% piperidine/NMP*	--	--	--	--	2 x 250
NMP for rinsing*	--	--	--	--	250(per wash)
1% Trifluoroacetic acid/DCM	--	--	--	--	3 X 400
Pyridine	79.10	--	150	--	3 X 4
Ethanol, denatured	--	--	--	--	300
Water	--	--	--	--	300

*per coupling cycle

Theoretical Yield: 44.4 g

<u>**Expected Yield**</u>: 90-105%
<u>**Actual Yield**</u>: 46.9 (105%)

<u>**Procedure:**</u>

The procedure used in the Example presented in Section 7.1., above, was repeated using 25.0g Fmoc-Leu-2-chlorotrityl resin, the required Fmoc-protected amino acids, and the solvent volumes indicated in the materials section above.

Following the final coupling reaction, the resin was washed 4 X 250 mL (10 vol) of NMP, then with 4 X 250 mL (10 vol) of DCM.

The peptide was cleaved from the resin using 3 X 400 mL (~15 vol) of 1 % TFA in DCM, followed by 1 X 200 mL (7.5 vol) of DCM. The cleavage fractions were collected onto pyridine (1:1 volume ratio to TFA), then the fractions and wash were analyzed for product content. The fractions containing product were combined and concentrated under vacuum to a volume of ~100 mL, then reconstituted with 300 mL of ethanol. The concentration was continued to remove residual DCM to a final volume of ~250 mL. To the stirred solution was added 300 mL of water to precipitate the product. The solids were collected by vacuum filtration and washed with ~50 ML of water. The product was air dried to give 46.4 g(105%) of Fmoc-AA(17-26)-OH of 97% HPLC purity.

Column: Phenomenox Jupiter C18
Flow rate: 1 mL/min
Detection: UV at 260 nm

Mobile phase: A: 0.1 % aqueous TFA
B: 0.1 % TFA in acetonitrile gradient from 75% B to 99% B in 20 minutes

Retention time: ~25 minutes

7.6 Preparation of Fragment Fmoc-AA(27-35)-OH (Fragment 16b)

Structure:

Fmoc-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(trt)-Trp(Boc)-OH (SEQ ID NO:17)

$C_{121}H_{148}N_{14}O_{24}$
MW 2182.61

Materials:	MW	eq	mmoles	grams	mL
Fmoc-Trp(Boc)-2-chlorotrityl resin	--	1.0	22.4	33.0	--
Fmoc-amino acid*	--	1.5	33.6	--	--
1-Hydroxybenzotriazole(HOBT) hydrate*	153.15	1.5	33.6	5.1	--
O-Benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate(HBTU)*	379.25	1.5	33.6	12.7	--
Diisopropylethylamine(DIEA)*	129.25	1.5	33.6	4.3	5.8
N-methyl-2-pyrrolidinone(NMP)*	--	--	--	--	225
Methylene chloride(DCM)*	--	--	--	--	75
20% piperidine/NMP*	--	--	--	--	2 x 250
NMP for rinsing*	--	--	--	--	250(per wash)
Trifluoronthanol	--	--	--	--	30
DCM for cleavage	--	--	--	--	240
Ethanol, denatured	--	--	--	--	300, 100
Water	--	--	--	--	300, 150
1% Trifluoroaetic acid(TFA) in DCM	--	--	--	--	2 X 250
Pyridine	79.10	--	--	--	2 X 2.5

*per coupling cycle

Theoretical Yield: 48.9 g   **Expected Yield:** 85-90%

Procedure:

The procedure used in the Example presented in Section 7.1, above, was repeated using 33.0 g Fmoc-Trp(Boc)-2-chlorotrityl resin, the required Fmoc-protected amino acids, and the materials indicated in the Materials section above.

Following the final coupling reaction, the resin was washed 4 X 250 mL (7.5 vol) of NMP, then with 4 X 250 mL (7.5 vol) of DCM.

The peptide was cleaved from the resin by treatment with 300 mL (~10 vol) of a solution of 8:1:1 DCM:TFE:HOAc for 2 hrs. The resin was drained and washed with 2 X 250 mL of DCM. The cleavage solution and washes were combined and concentrated to a volume of ~50 mL, then reconstituted with

250 mL of ethanol. The solution was chilled with stirring in an ice bath to 0-5°C. To the stirred solution was added 125 mL of water to precipitate the product. The solids were collected by vacuum filtration and washed with ~50 mL of water. The product was air dried to give 32.0 g(65.4%) of Fmoc-AA(27-35)-OH of 95% HPLC purity.

The resin was treated with 2 X 250 mL of a 1% solution of TFA in DCM followed by a wash with 100 mL of DCM. The cleavage fractions were collected onto pyridine in a 1:1 volume ratio with TFA. The combined eluants and wash were concentrated to ~50 mL volume. To the solution was added 100 mL ethanol, then 150 mL of water. The product slurry was vacuum filtered yielding a second crop of 10.7 g (21.9%)(95% HPLC purity) to give a combined yield of 87.3%. The 1% TFA/DCM cleavage is preferred due to its greater effectiveness and lower volumes.

Column: Phenomenox Jupiter C5, 300Å, 5μ
Flow rate: 0.75 mL/min
Detection: UV at 260 nm

Mobile phase: A: 0.05% aqueous TFA
B: 0.1% TFA in 1:1
IPA:MeOH gradient
from
70% B to 97% B in 10 minutes

Retention time: ~25 minutes

8. EXAMPLE: SOLUTION PHASE SYNTHESIS OF PEPTIDE FRAGMENTS

Presented below, in Sections 8.1 - 8.11, are examples of the solution phase synthesis of peptide intermediates as listed in Tables 1, 2, and/or 3.

8.1 Preparation of Fragment Fmoc-AA9-16OPNB by Coupling of Para Nitrobenzylester (OPNB) Of Glutamine to Fmoc-AA9-15OH

Structure:

Fmoc-Ile-Glu(OtBu)-Glu(OtBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-OPNB (SEQ ID NO:7)

$C_{129}H_{141}N_{13}O_{22}$MW 2227.65

Materials:	MW	eq	mmoles	grams	mL
FmocAA9-15OH	1963.39	1	9.7	19	--
HBrGlnOPNB (BaChem,#509709)	362.19	1.1	10.6	3.85	--
HOAT	136	1.1	10.6	1.45	--
HBTU	379.25	1.1	10.6	4.04	--
$EtPr_2N$ (d=0.755)	129.25	2.1	20.3	2.62	3.48
NMP	--	--	--	--	200
0.5N HCl	--	--	--	--	250
ethyl acetate	--	--	--	--	250
hexane	--	--	--	--	250

Theoretical Yield: 21.5 g **Expected Yield:** 90-105%

Procedure:

FmocAA9-15OH (as synthesized in Section 7.2, above), HBrGlnOPNB, HOAT and $EtPr_2N$ were combined in a 1L round bottom flask containing a magnetic stir bar and NMP (200 mL) was added. The resulting solution was placed under a nitrogen atmosphere and cooled to 0-5°C with stirring. To the cool solution was added HBTU. The solution was stirred for 15 minutes at 0-5°C, the ice bath was removed and stirring was continued for 2.5 hours (note 1).

The reaction mixture was cooled to 0-5°C, and 0.5N aqueous HCl (250 mL) was added to precipitate the protected peptide. The solids were collected by vacuum filtration and dried in the filter flask to yield 24 g of crude FmocAA9-16OPNB. The solid was dissolved in ethyl acetate (250 mL), dried over magnesium sulfate (10g), filtered and concentrated to a volume of 100 mL. The solution was cooled to 0-5°C and hexane (250 mL) was added to precipitate the peptide. The solid was collected by vacuum filtration and dried providing

21.5g of FmocAA9-16OPNB in 100% yield and 91-94% HPLC purity (note 2).

Notes:

1. In process control (IPC) by thin layer chromatography (TLC).

    90/10 chloroform/ethanol
    UV, Iodine detection
    Rt FmocAA9-15OH 0.46
    Rt FmocAA9-16OPNB 0.57

2. Phenomenex Jupiter, C5, 5μ, 300A

    0.75 mL/min, 260 nm
    A H_2O/0.05% TFA
    B 50% IPA/MeOH/0.05% TFA
    70-97%B over 10 min, 97%B for 8 min.
    Retention time: 13.3 minutes

8.2 Preparation of Fragment HCl HAA9-16OPNB

Structure:

HCl H-Ile-Glu(OtBu)-Glu(OtBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-OPNB (SEQ ID NO:7)

$C_{144}H_{131}ClN_{13}O_{20}$
MW 2041.02

Materials:	MW	eq	mmoles	grams	mL
FmocAA9-16OPNB	2227.65	1	9.43	21	--
Piperidine	--	--	--	--	10
THF	--	--	--	--	190
Methyl tertbutyl ether (MTBE)	--	--	--	--	250
Hexane	--	--	--	--	350
Methanol	--	--	--	--	150
0.5N HCl	--	--	--	--	100
2-propanol	--	--	--	--	50

Theoretical Yield: 19.2 g   **Expected Yield:** 85-105%

Procedure:

A 1 L round bottom flask containing a magnetic stir bar was charged with FmocAA9-16OPNB (as synthesized in Section 8.1, above) and 20:1 tetrahyrofuran/piperidine. The resulting solution was stirred under an atmosphere of nitrogen at room temperature for 60 minutes (note 1). Hexane (350 mL) was added to precipitate the peptide. The solvent was decanted from the sticky solid. The solid was triturated with MTBE (200 mL) at room temperature for 18 hours. The solid was collected by vacuum filtration and dried to give 18.9g of HAA9-16OPNB (note 2).

The solid was dissolved in methanol (150 mL), cooled to 0-5°C with stirring. 0.5N aqueous hydrochloric acid (100 mL) was added to precipitate the peptide. The solids were collected by vacuum filtration, washed with water (50 mL) then 2-propanol (50 mL) and dried to give 17.7g of HCl HAA9-16OPBN in 92% yield and an HPLC purity of 92A% (note 3).

Notes:

1. In process control, HPLC
   Phenomenex Jupiter, C5, 5μ, 300A
   0.75 mL/min, 260 nm
   A H_2O/0.05% TFA
   B 50% IPA/MeOH/0.05% TFA
   70-97%B over 10 min, 97%B for 8 min.
   Retention time: FmocAA9-16OPNB, 13.3 minutes; HAA9-16OPNB, 10.7 minutes

2. HAA9-16OPNB isolated at this point contains trace amounts of piperidine and the benzylfulvene piperidine adduct. Both are removed before coupling with RAA1-8OH.

3. Phenomenex Jupiter, C5, 5μ, 300A
   0.75 mL/min, 260 nm
   A H_2O/0.05% TFA

B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 min, 100%B for 5 min.
Retention time: HAA9-16OPNB, 7.2 minutes

TLC conditions:
90/10 dichloromethane/ethanol
UV, iodine detection
Rf: HAA9-16OPNB, 0.64

8.3 Preparation of Fragment AcAA1-16OPNB by Solution-Phase Coupling of Fragments AcAA1-8OH and HAA9-16OPNB

Structure:

Ac-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(OtBu)-Glu(OtBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-OPNB (SEQ ID NO:4)

$C_{194}H_{246}N_{23}O_{34}$MW 3427.43

Materials:	MW	eq	mmoles	grams	mL
AcAA1-8OH	1440.86	1.3	0.13	0.186	--
HCl HAA9-16OPNB	2041.02	1	0.10	0.204	--
HBTU	379.25	1.1	0.11	0.042	--
HOAT	136.1	1.1	0.11	0.015	--
$EtPr_2N$	129.25	2.1	0.21	0.027	0.036
DMF	--	--	--	--	4.5
DMSO	--	--	--	--	0.5
water	--	--	--	--	7
MTBE	--	--	--	--	3.5

Theoretical Yield: 0.38 **Expected Yield:** 85-105%

Procedure:

A 25 mL round bottom flask containing a magnetic stir bar was charged with AA1-8OH (as synthesized in Section 7.1, above), HCl HAA9-16OPNB (as synthesized in Section 8.2, above) and HOAT. The solids were dissolved in 9:1 DMF:DMSO (5 mL) containing $EtPr_2N$, then cooled to 0-5°C under an atmosphere of nitrogen (note 1). To the cool solution was added HBTU. The reaction mixture was stirred at 0-5°C for 15 minutes, then warmed to room temperature and stirred an additional 60 minutes (note 2). The peptide was precipitated

from the solution by addition of water (7 mL). The solids were collected by vacuum filtration, washed with water (10 mL) and dried to give 0.36g of crude AcAA1-16OPNB. The solid was triturated with MTBE (3.5mL) for 1.5 hours at room temperature, collected by vacuum filtration and dried to give 0.335g of AcAA-16OPNB in 88% yield and 82A% HPLC purity (note 3).

Notes:

1 It is important that all the solids are in solution before cooling to 0-5°C and adding HBTU.

2 In process control, TLC, HPLC
Phenomenex Jupiter, C5, 5μ, 300A
0.75 mL/min, 260 nm
A H_2O/0.05% TFA
B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 min, 100%B for 5 min.
Retention time: HAA9-16OPNB, 7.2 minutes (Ac1-8OH has no absorbance at 260 nm).
Retention time: AcAA1-16OPNB, 12.45

TLC conditions
90/10 chloroform/ethanol
UV, iodine detection
Rf: HAA9-16OPNB, 0.64
Rf: Ac1-8OH, 0.35
Rf: Ac1-16OH, 0.48

3 Phenomenex Jupiter, C5, 5μ, 300A
0.75 mL/min, 260 nm
A H_2O/0.05% TFA
B 50% IPA/MeOH/0.05% TFA
70-97%B over 10 min, 97%B for 8 min
Retention time: Ac1-16OPNB, 16.4.

8.4 Preparation of Fragment FmocAA1-16OPNB by Solution-Phase Coupling of Fragments FmocAA1-8OH and HCl HAA9-16OPNB

Structure:

Fmoc-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(OtBu)-Glu(OtBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-OPNB (SEQ ID NO:4)

$C_{207}H_{253}N_{23}O_{34}$
MW 3607.49

Materials:	MW	eq	mmoles	grams	mL
FmocAA1-8OH	1620.92	1	7.84	12.7	--
HCl HAA9-16OPNB	2041.02	1	7.84	16.0	--
HBTU	379.25	1.2	9.42	3.57	--
HOAT	136.1	1.2	9.42	1.28	--
$EtPr_2N$ (d=0.755)	129.25	2.5	19.6	2.55	3.36
DMF	--	--	--	--	250
10% sodium chloride/water (wt/vol)	--	--	--	--	450
methanol	--	--	--	--	200
water	--	--	--	--	100

Theoretical Yield: 28.3 g **Expected Yield:** 85-100%

Procedure:

A 1L round bottom flask containing a magnetic stir bar was charged with FmocAA1-8OH (as synthesized in Section 7.1, above), HCl HAA9-16OPNB (as synthesized in Section 8.2, above), HOAT and DMF (250 mL). To the solution was added $EtPr_2N$. The solution was cooled to 0-5°C and HBTU was added. The reaction mixture was stirred at 0-5°C for 15 minutes, then warmed to room temperature and stirred an additional 70 minutes (note 1). The reaction mixture was cooled to 0-5°C and 10% sodium chloride/water (200 mL) was added to precipitate the peptide. The solids were collected by vacuum filtration, washed with water (50 mL) and dried to give 27g of crude FmocAA1-16OPNB. The solid was dissolved in methanol (200mL) and added to a stirred solution of sodium chloride in water (10% wt/vol, 300 mL). The solids were collected by vacuum filtration, washed with water (50 mL) and dried to give 26g of FmocAA1-16OPNB in 92% yield and 90A% purity by HPLC (note 1).

Notes:

1 In process control, HPLC
Phenomenex Jupiter, C5, 5μ, 300A
0.75 mL/min, 260 nm
A H_2O/0.05% TFA
B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 min, 100%B for 5 min.
Retention time: HAA9-16OPNB, 7.2 minutes, FmocAA1-8OH, 7.9 minutes
Retention time: FmocAA1-16OPNB, 14.5 minutes

8.5 Preparation of Fragment H-AA1-16OPNB

Structure:

H-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(tBu)-Glu(tBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-OPNB (SEQ ID NO:4)

$C_{192}H_{243}N_{23}O_{32}$
MW 3384.41

Materials:	MW	eq	mmoles	grams	mL
FmocAA1-16OPNB	3607.49	1	0.28	1.0	--
piperidine	--	--	--	--	0.6
dichloromethane	--	--	--	--	11.4
hexane	--	--	--	--	45

Theoretical Yield: 0.94 g **Expected Yield:** 90-105%

Procedure:

A 50 mL round bottom flask containing a magnetic stir bar was charged with FmocAA1-16OPNB (as synthesized in Section 8.4, above), dichloromethane (11.4 mL) and piperidine (0.6 mL). The solution was stirred at room temperature under an atmosphere of nitrogen for 90 minutes (note 1). Hexane (45 mL) was added to the reaction mixture and the solvent

volume was reduced to 25 mL by vacuum distillation. The resulting solids were collected by vacuum filtration and dried to give 0.96g of HAA1-16OPNB in 102% yield. HPLC analysis of the solid indicated 72A% HAA1-16OPNB and 18A% fulvene and piperidine-fulvene adduct.

Notes:

1 Phenomenex Jupiter, C5, 5μ, 300A
0.8 mL/min, 260 nm
A H_2O/0-05% TFA
B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 min, 100%B for 5 min
Retention time FmocAA1-16OPNB, 14.1 min
Retention time HAA1-16OPNB, 11.6 min
Retention time fulvene and piperdine-fulvene adduct, 5.5 and 4.8 min.

8.6 Preparation of Fragment Ac-AA1-16OPNB by Acetylation of N-terminus of HAA1-16OPNB

Structure:
Ac-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(tBu)-Glu(tBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-OPNB (SEQ ID NO:4)

$C_{194}H_{246}N_{23}O_{34}$
MW 3427.43

Materials:	MW	eq	mmoles	grams	mL
HAA1-16OPNB	3384.41	1	0.28	0.95	--
acetic anhydride (d=1.08)	102.09	3	0.84	0.086	0.080
pyridine (d=0.978)	79.1	3	0.84	0.67	0.068
DMF	--	--	--	--	10
water	--	--	--	--	30
MTBE	--	--	--	--	10
hexane	--	--	--	--	10

Theoretical Yield: 0.96 g   **Expected Yield:** 80-100%

Procedure:

A 50 mL round bottom flask containing a magnetic stir bar was charged with HAA1-16OPNB (as synthesized in Section 8.5, above), DMF (10 mL), acetic anhydride and pyridine. The reaction mixture was stirred at room temperature under an atmosphere of nitrogen for 60 min (note 1). Water (20 mL) was added to precipitate the peptide. The solids were collected by vacuum filtration, washed with water (10 mL) and dried to give 0.87g of AcAA1-16OPNB. To remove residual fulvene and piperidine-fulvene adduct, the solid was triturated with 1:1 MTBE/hexane (20 mL) for 4.5 hours at room temperature. The solids were collected by vacuum filtration and dried to give 0.82 g of AcAA1-16OPNB in 85% yield and over 90A% purity by HPLC (note 1).

Notes:

1 In process control, HPLC
Phenomenex Jupiter, C5, 5μ, 300A
0.8 mL/min, 260 nm
A H_2O/0.05% TFA
B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 min, 100%B for 15 min
Retention time HAA1-16OPNB, 11.6 min
Retention time AcAA1-16OPNB, 12.1 min

TLC, 10% ethanol in dichloromethane
UV, Iodine detection
Rf AcAA1-16OPNB, 0.69

8.7 Preparation of Fragment AcAA1-16OH by Selective Removal of a Paranitrobenzyl Protecting Group From AcAA1-16OPNB in the Presence of His(trt).

Structure:

Ac-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(OtBu)-Glu(OtBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-OH (SEQ ID NO:4)

$C_{187}H_{241}N_{22}O_{32}$
MW 3276.4

Materials:	MW	eq	mmoles	grams	mL
AcAA1-16OPNB	3424.86	1	0.23	0.80	--
10% Pd/C, Degussa, 50% water	--	--	--	0.30	--
ammonium formate	63.06	15	3.5	0.22	--
DMF	--	--	--	--	15
Water	--	--	--	--	120
Ethyl acetate	--	--	--	--	100
Hexane	--	--	--	--	44
methanol	--	--	--	--	10
saturated aq.NaCl	--	--	--	--	5
MTBE	--	--	--	--	4

Theoretical Yield: 0.76 g **Expected Yield:** 70-85%

Procedure:

A 25 mL round bottom flask containing a magnetic stir bar was charged with AcAA1-16OPNB (as synthesized in Section 8.6, above) sand DMF (10 mL). To this solution was added a solution of ammonium formate in water (0.5 mL), then wet palladium on carbon (Degussa, 10%, 50% water). The slurry was stirred under an atmosphere of nitrogen at room temperature for 120 minutes (note 1). The slurry was filtered through a tightly packed bed of celite into 90 mL of water. The filter cake was washed with DMF (5 mL). The aqueous suspension was washed with ethyl acetate (100 mL). The ethyl acetate was then concentrated to a volume of 20 mL (note 2). Hexane (40 mL) was added to complete the precipitation and the solvent was decanted from the solids. The solids were dissolved in methanol (10 mL) and 4:1 water/saturated aqueous sodium chloride (25 mL) was added to precipitate the peptide. The solids were collected by vacuum filtration, washed with water (10 mL) and dried to give 0.62g

of AcAA1-16OH. The solids were triturated with 50% MTBE/hexane (8 mL) at room temperature for 15 hours, collected and dried to give 0.59g of AcAA1-16OH in 77% yield and 90A% purity by HPLC (note 3).

Notes:

1 In process control, TLC
80/20 dichloromethane/ethanol
UV, iodine detection
Rf: AcAA1-16OPNB, 0.90
Rf: Ac1-16OH, 69

2 The AcAA1-16OH may begin to precipitate as the solvent volume is reduced.

3 Phenomenex Jupiter, C5, 5μ, 300A
0.8 mL/min, 260 nm
A H_2O/0.05% TFA
B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 minutes, 100%B for 5 minutes
Retention time: AcAA1-16OH, 10.73 minutes.

8.8 Preparation of Fragment FmocAA27-36NH_2 by Solution-Phase Coupling of FmocAA27-35OH With HPheNH_2

Structure:

Fmoc-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(trt)-Trp(Boc)-Phe-NH_2 (SEQ ID NO:18)
$C_{130}H_{159}N_{16}O_{24}$
MW 2329.64

Materials:		MW	eq	mmoles	grams	mL
FmocAA27-35OH		2182.61	1	18.4	40.2	--
HPheNH_2		162.21	1.2	22.1	3.6	
HBTU		379.25	1.2	22.1	8.4	
HOAT		136.1	1.2	22.1	3.0	
$EtPr_2N$	(d=0.755)	129.25	2.1	38.7	5.0	6.6
DMF						500

Water 600

Theoretical Yield 42.8g **Expected Yield:** 90-105%

Procedure:

A 2L round bottom flask containing a magnetic stir bar was charged with FmocAA27-35OH (as synthesized in Section 7.6, above), HOAT, $HpheNH_2$ and DMF (500 mL). $EtPr_2N$ was added and the solution was cooled to 0-5°C then HBTU was added. The reaction mixture was stirred for 15 minutes at 0-5°C then warmed to room temperature and stirred an additional 70 minutes (note 1). The solution was cooled to 0-5°C and water (500 mL) was added to precipitate the peptide. The solids were collected by vacuum filtration, washed with water (100 mL) and dried to give 43g of FmocAA27-$36NH_2$ in 100% yield and 93A% purity by HPLC (note 2).

Notes:

1 In process control, TLC
88/12 dichloromethane/methanol
UV, iodine detection
Rf: FmocAA27-35OH, 0.49
Rf: FmocAA27-$36NH_2$, 0.63

2 Phenomenex Jupiter, C18, 5μ, 300A.
1.0 mL/min, 260 nm
A H_2O/0.1 % TFA
B ACN
75-99%B over 20 min, 99%B for 5 min.
Retention time: 29.4 minutes

8.9 Preparation of Fragment H-AA(27-36)-NH_2

Structure:

H-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(trt)-Trp(Boc)-NH_2 (SEQ ID NO:17)

$C_{115}H_{148}N_{16}O_{22}$
MW 2106.56

Materials:	MW	eq	mmoles	grams	mL
FmocAA(27-36)-NH_2	2328.8	1.0	9.3	21.7	-
Piperidine	85.15	5.0	46.6	4.0	4.6
Methylene chloride (DCM)	-	-	-	-	100
Water	-	-	-	-	2 X 100
Methyl t-butyl ether (MTBE)	-	-	-	-	100 + 30

Theoretical Yield 19.6g **Expected Yield**: 85-95%

Procedure:

To a 250 mL round bottom flask equipped with a magnetic stirrer and nitrogen blanket was charged the Fmoc-fragment synthesized in Section 8.8, above, the methylene chloride (~5 vol), and the piperidine. A solution was obtained and stirred at room temperature for 1.5 hours (Note 1).

The solution was washed with 2 X 100 mL of water. The layers were separated and the organic layer was concentrated under vacuum to approximately one half the original volume. MTBE was added portionwise, 2 X 50 mL, while the concentration was continued to remove DCM to a point of heavy precipitation and a final pot volume of approximately 150 mL.

The product slurry was stirred and chilled in an ice bath at 0-5°C for approximately one hour. The solids were isolated by vacuum filtration and washed with 2 X 15 mL of MTBE. The product was air dried to give 17.6 g (89.6%) of H-AA(27-36)NH_2 of 95% HPLC purity (Note 2).

Notes:

1) Reaction completion is monitored by HPLC:

Column: Phenomenox Jupiter C18; 300Å; 5μ
Flow rate: 1 mL/min
Detection: UV at 260 nm
Mobile phase: A: 0.1% aqueous TFA
B: 0.1% TFA in acetonitrile

gradient from 75% B to 99% B in 20 minutes

Retention time: approximately 18 minutes

2) Both of the Fmoc by-products, the dibenzofulvene and its piperidine adduct, are effectively removed in the workup; however, a use-test should be performed before carrying the material forward. If the material fails the use test, the workup procedure is repeated by dissolving the solid in DCM (5 vol), then continuing with the process described above.

8.10 Preparation of Fragment FmocAA17-36NH_2 by Solution-phase coupling of FmocAA17-26OH with HAA27-36NH_2

Structure:

Fmoc-Glu(OtBu)-Lys(Boc)-Asn(trt)-Glu(OtBu)-Gln(trt)-Glu(OtBu)-Leu-Leu-Glu(OtBu)-Leu-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(trt)-Trp(Boc)-Phe-NH_2 (SEQ ID NO:12)

$C_{242}H_{313}N_{29}O_{46}$
MW 4364.38

Materials:		MW	eq	mmoles	grams	mL
FmocAA17-26OH		2275.82	1	9.5	21.6	
HAA27-36NH_2		2106.56	1	9.5	20	
HOAT		136.1	1.2	11.4	1.55	
HBTU		379.25	1.2	11.4	4.33	
$EtPr_2N$	(d=0.755)	129.25	2	19.0	2.46	3.25
DMF						400
Water						600
2-propanol						1100

Theoretical Yield 41.5g **Expected Yield:** 80-85%

Procedure:

A 2L round bottom flask containing a magnetic stir bar was charged with FmocAA17-26OH (as synthesized in Section 7.5, above), HAA27-36NH_2 (as synthesized in Section 8.9, above), HOAT and DMF (400 mL). $EtPr_2N$ was added, the stirred solution was cooled to 0-5°C under an atmosphere of nitrogen and HBTU was added. The reaction mixture was stirred at 0-5°C for 15 minutes, then warmed to room temperature and stirred an additional 2.5 hours (note 1). Water (500 mL) was

added to the reaction mixture to precipitate the peptide (note 2). The resulting slurry was stirred for 45 minutes, the solids were collected by vacuum filtration, washed with water (100 mL) and dried. The solids were returned to the 2L round bottom flask containing a magnetic stir bar and 60°C 2-propanol (1.1 L) was added. The slurry was stirred under an atmosphere of nitrogen as it cooled to room temperature (overnight). The solids were collected by vacuum filtration and dried to give 37.5g of FmocAA17-36NH_2 in 90% yield and 95.5A% purity by HPLC (note 1).

Notes:

1 In process control, HPLC
Phenomenex Jupiter, C5, 5μ, 300A
0.75 mL/min, 260 nm
A H_2O/0.05% TFA
B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 min, 100%B for 25 min.
Retention time: FmocAA17-26OH, 8.2 minutes, HAA26-36NH_2, 8.4 minutes
Retention time: FmocAA17-36NH_2, 13.3 minutes

2 The reaction mixture warmed to 38°C on addition of the water.

8.11 Preparation of Fragment H-AA(17-36)-NH_2

Structure:

H-Glu(OtBu)-Lys(Boc)-Asn(trt)-Glu(OtBu)-Gln(trt)-Glu(OtBu)-Leu-Leu-Glu(OtBu)-Leu-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(trt)-Trp(Boc)-NH_2 (SEQ ID NO:19)

$C_{227}H_{303}N_{29}O_{44}$ HCl

Materials:	MW	eq	mmoles	grams	mL
FmocAA17-(17-36)-NH_2	4364.36	1.0	5.2	22.5	-
5 N NaOH(ag)	-	-	-	-	55
Tetrahydrofuran (THF)	-	-	-	-	170
1 N HC1	-	-	-	-	13
Saturated NaCL(aq)	-	-	-	-	2 X 55
Heptane	-	-	-	-	3 X 25 + 50, 200 + 50

Theoretical Yield 21.6g **Expected Yield:** 95-100%

Procedure:

To a 250 mL round bottom flask equipped with an air stirrer and nitrogen blanket was charged with the Fmoc - fragment synthesized in Section 8.10, above, the THF (approximately 7.5 vol), and the 5 N NaOH(~2.5 vol). A two-phase solution was obtained and stirred at room temperature for 10-15 minutes (Note 1).

The layers were separated and the organic phase was adjusted to pH 2-3 with 1 N HCl. The solution was then washed 2 X 55 mL (2.5 vol) with a saturated brine solution (Note 2). The layers were separated and the organic layer was concentrated under vacuum at 15-20°C to about ½ original volume. Heptane was added portionwise, 3 X 25 mL, while the concentration was continued to remove THF to a point of heavy precipitation and a final pot volume of approximately 100 mL (Note 2).

The product slurry was stirred at room temperature for about 2 hrs. The solids were isolated by vacuum filtration and washed with about 50 mL of heptane. The product was air dried to give 21.0g (97.5%) of H-AA(17-36)NH_2.

A rework may be performed to remove residual dibenzofulvene byproduct. The product was slurried at room temperature in 200 mL of heptane for 3 hrs. The material was filtered, washed with about 50 mL of heptane, and air dried yielding 20.8 g (96.6%) of product of over 95% HPLC purity.

Notes:

1) Reaction completion is monitored by HPLC:

Column: Zorbax LP C8; 1 OOA; 2011 Flow rate: 1 mL/min
Detection: UV at 260 nm
Mobile phase: A: 0. 1 %aqueous TFA
B: 0.05% TFA in 1:1 ACN:IPA
gradient from 80% B to 99% B in 20 minutes
Retention time: approximately 18 minutes

2) The solids precipitate initially as a waxy gum which remains stirrable and triturates with further concentration to a filterable solid.

9. EXAMPLE: Synthesis of Full Length T-20 peptides

Presented herein, in Sections 9.1 - 9.5, below, are examples of the utilization of the peptide intermediate fragments to produce full length T-20 peptides.

The Example presented in this Section demonstrates the successful coupling of solid phase and solution phase synthesis techniques to produce a full-length T-20 peptide from peptide intermediate fragments.

9.1 Preparation of Fragment AcAA1-36NH_2 by Solution-phase coupling of AcAA1-16OH with HAA17-36NH_2

The synthesis route described here represents the culmination of the T-20 four fragment approaches schematically depicted in FIGS. 1 and 3. In instances in which AcAA1-16OH is synthesized via solid phase techniques, the approach in FIG. 1 is followed, and in instances in which AcAA1-16 OH is synthesized via solution phase techniques, the approach in FIG. 3 is followed. It is noted that the T-20 full length peptide synthesized here has the amino acid sequence of SEQ ID NO:1, with an acetyl modification at its amino terminus (i.e., "X") and an amido modification at its carboxyl terminus (i.e., "Z").

Structure:

Ac-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(OtBu)-Glu(OtBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-Glu(OtBu)-Lys(Boc)-Asn(trt)-Glu(OtBu)-Gln(trt)-Glu(OtBu)-Leu-Leu-Glu(OtBu)-Leu-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(trt)-Trp(Boc)-Phe-NH_2 (SEQ ID NO:1)

$C_{414}H_{543}N_{51}O_{74}$
MW 7411.95

Materials:		MW	eq	mmoles	grams	mL
AcAA1-1OH		3276.4	1	0.24	0.79	
HClHAA17-36NH_2		4178.56	1	0.24	1.0	
HOAT		136.1	1.1	0.26	0.036	
HBTU		379.25	1.0	0.95	0.25	
$EtPr_2N$	(d=0.755)	129.25	2.8	0.67	0.087	0.115
DMF						20
water						25
saturated NaCl						5
MTBE						10
hexane						10

Theoretical Yield 1.77g **Expected Yield:** 85-100%

Procedure:

A 100 mL round bottom flask containing a magnetic stir bar was charged with AcAA1-16OH (as synthesized in either Section 7.4, above, via solid phase techniques or in Section 8.7 above, via solution phase techniques), HOAT, DMF (20 mL) then $EtPr_2N$ (0.074 mL). The solution was cooled to 0-5°C under an atmosphere of nitrogen and HBTU was added. The solution was stirred for 15 minutes at 0-5°C and HClHAA17-36NH_2 (as synthesized in Section 8.11, above) was added, followed an additional 0.041 mL of $EtPr_2N$. The cooling bath was removed and the reaction mixture was stirred for 2 hours (note 1). To precipitate the peptide, water (25 mL) and saturated aqueous sodium chloride (5 mL) was added. The solids were collected by vacuum filtration, washed with water (10 mL) and dried to give 1.74g of crude AcAA1-36NH_2 (note 2). The solids were triturated with 50% MTBE/hexane at room

temperature for 2.5 hours, collected by vacuum filtration and dried to give 1.70g in 96% yield and 92A% purity by HPLC.

Notes:

1) In process control, HPLC
Phenomenex Jupiter, C5, 5μ 300A
0.8 mL/min, 260 nm
A H_2O/0.05% TFA
B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 minutes, 100%B for 15 minutes
Retention time: AcAA1-16OH, 11.8 minutes.
Retention time: HCl HAA17-36NH_2, 12.7 minutes.
Retention time: AcAA1-36NH_2, 22.9 minutes.

2) The water dropout produces a very fine precipitate. A double filtration may be required.

9.2 Preparation of Fragment FmocAA1-36NH_2 (T-20) by Solution-phase coupling of FmocAA1-16OH with HAA17-36NH2

The Example presented in this Section demonstrates the successful coupling of solid and liquid phase synthesis techniques to produce a T-20 peptide from peptide intermediate fragments. In particular, the synthesis route described here represents the T-20 three fragment approach schematically depicted in FIG. 4 to the point in the figure at which FmocAA1-36NH_2 is synthesized.

Structure:

Fmoc-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(OtBu)-Glu(OtBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-Glu(OtBu)-Lys(Boc)-Asn(trt)-Glu(OtBu)-Gln(trt)-

Glu(OtBu)-Leu-Leu-Glu(OtBu)-Leu-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(trt)-Trp(Boc)-Phe-NH_2
(SEQ ID NO:1)

$C_{427}H_{551}N_{51}O_{75}$
MW 7593.01

Materials:		MW	eq	mmoles	grams	mL
FmocAA1-16OH		3453.89	1	0.12	0.41	
HCl HAA17-36NH_2		4174.88	1	0.12	0.50	
HOAT		136.1	1.25	0.15	0.020	
HBTU		379.25	1.25	0.15	0.057	
$EtPr_2N$	(d=0.755)	129.25	2.5	0.30	0.039	0.051
DMF						10
water						18
2-propanol						14
Theoretical Yield	0.91g	**Expected Yield:**		85-100%		

Procedure:

A 25 mL round bottom flask containing a magnetic stir bar was charged with FmocAA1-16OH (as synthesized in Section 7.3, above), HCl HAA17-36NH_2 (as synthesized in Section 8.11, above), HOAT, DMF (10 mL) the $EtPr_2N$ was added. The solution was cooled to 0-5°C under an atmosphere of nitrogen and HBTU was added. The reaction mixture was stirred at 0-5°C for 15 minutes, then warmed to room temperature and stirred for 1.5 hours (note 1). Water was added to precipitate the peptide and the solids were collected by vacuum filtration and dried. The solids were triturated with 2 propanol (14 mL) for 15 hours at room temperature then water (3 mL) was added to drive the desired product from solution. The solids were collected by vacuum filtration and dried to give 0.80g of FmocAA1-36NH_2 in 88% yield and 85A% HPLC purity.

Notes:

1) In process control, HPLC
Phenomenex Jupiter, C5, 5μ, 300A
0.8 mL/min, 260 nm
A H_2O/0.05% TFA

B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 minutes, 100%B for 15 minutes
Retention time: FmocAA1-16OH, 11.4 minutes.
Retention tim: HCl HAA17-36NH_2, 12. minutes.
Retention time: FmocAA1-36NH_2, 20.4 minutes.

TLC, 9/1 dichloromethane/ethanol
UV, Iodine detection
Rft: FmocAA1-36NH_2, 0.71.

9.3 Preparation of Fragment HAA1-36NH_2 (T-20)

The Example presented in this Section demonstrates the successful coupling of solid and liquid phase synthesis techniques to produce a T-20 peptide from peptide intermediate fragments. In particular, the synthesis route described here represents the T-20 three fragment approach schematically depicted in FIG. 4 to the point in the figure at which H-AA1-36-NH_2 is synthesized.

Structure:

H-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(OtBu)-Glu(OtBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-Glu(OtBu)-Lys(Boc)-Asn(trt)-Glu(OtBu)-Gln(trt)-Glu(OtBu)-Leu-Leu-Glu(OtBu)-Leu-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(trt)-Trp(Boc)-Phe-NH_2 (SEQ ID NO:1)

$C_{412}H_{541}N_{51}O_{73}$
MW 7370.94

Materials:	MW	eq	mmoles	grams	mL
FmocAA1-36NH_2	7593.01	1	0.105	0.80	
piperidine					0.5
DMF					9.5
water					20
saturated aqueous NaCl					5
MTBE					5
hexane					5

Theoretical Yield .77g **Expected Yield:** 85-95%

Procedure:

A 25 mL round bottom flask containing a magnetic stir bar was charged with FmocAA1-36NH_2 (as synthesized in Section 9.2, above), DMF (9.5 mL) and piperidine (0.5 mL). The solution was stirred at room temperature under an atmosphere of nitrogen for 2 hours (note 1, 2). Water (20 mL) and saturated aqueous sodium chloride (5 mL) was added to precipitate the protected peptide. The solids were collected by vacuum filtration and dried to give 0.77g of HAA1-36NH_2 contaminated with fulvene and the piperidine-fulvene adduct. The solids were triturated with 50% MTBE/hexane at room temperature for 15 hours to remove the fulvene and the piperidine-fulvene adduct. The solids were collected by vacuum filtration and dried to give 0.73g of HAA1-36NH_2 in 95% yield and 90A% purity by HPLC.

Notes:

1) In process control, HPLC
Phenomenex Jupiter, C5, 5μ, 300A
0.8 mL/min, 260 nm
A H_2O/0.05% TFA
B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 minutes, 100%B for 15 minutes
Retention time: FmocAA1-36OH, 20.4 minutes.
Retention time: HCl HAA1-36NH_2, 19.9 minutes
Retention time: fulvene and piperidine-fulvene adduct, 5 minutes.

TLC, 9/1 dichloromethane/ethanol
UV, Iodine detection
Rt: FmocAA1-36NH_2, 0.71.

2) The product and starting material do not separate well on TLC or reverse phase HPLC. Product formation was

followed by observing the fulvene and piperidiine-fulvene adduct.

9.4 Preparation of Fragment AcAA1-36NH$_2$ (T-20) by N-terminal acetylation of HAA1-36NH$_2$

The Example presented in this Section demonstrates the successful coupling of solid and liquid phase synthesis techniques to produce a T-20 peptide from peptide intermediate fragments. In particular, the synthesis route described here represents the T-20 three fragment approach schematically depicted in FIG. 4 to the point in the figure at which Ac-AA1-36-NH$_2$ is synthesized.

Structure:

Ac-Tyr(tBu)-Thr(tBu)-Ser(tBu)-Leu-Ile-His(trt)-Ser(tBu)-Leu-Ile-Glu(OtBu)-Glu(OtBu)-Ser(tBu)-Gln(trt)-Asn(trt)-Gln(trt)-Gln-Glu(OtBu)-Lys(Boc)-Asn(trt)-Glu(OtBu)-Gln(trt)-Glu(OtBu)-Leu-Leu-Glu(OtBu)-Leu-Asp(tBu)-Lys(Boc)-Trp(Boc)-Ala-Ser(tBu)-Leu-Trp(Boc)-Asn(trt)-Trp(Boc)-Phe-NH$_2$ (SEQ ID NO:1)

$C_{414}H_{543}N_{51}O_{74}$
MW 7411.95

Materials:	MW	eq	mmoles	grams	mL
HAA1-36NH$_2$	7370.94	1	0.096	0.71	
acetic anhydride (d=1.08)	102.09	3	0.29	0.029	0.027
pyridine (d=0.978)	79.1	6	0.58	0.046	0.046
DMF					10
water					17.5
saturated aqueous NaCl					7.5

Theoretical Yield 0.71g **Expected Yield:** 85-100%

Procedure:

A 25 mL round bottom flask containing a magnetic stir bar was charged with HAA1-36NH$_2$ (as synthesized in Section 9.3, above), DMF (10 mL), pyridine (0.046 mL) and acetic anhydride (0.027 mL) (note 1). The solution was stirred at

room temperature under an atmosphere of nitrogen for 4 hours (note 2). Water (7.5 mL) and saturated aqueous sodium chloride (7.5 mL) was added to precipitate the protected peptide. The solids were collected by vacuum filtration, washed with water (10 mL) and dried to give 0.65g of AcAA1-$36NH_2$ in 91% yield and 90A% purity by HPLC (note 3).

Notes:

1) Dichloromethane may be also utilized as the solvent for this reaction.

2) In process control, HPLC
Phenomenex Jupiter, C5, 5μ, 300A
0.8 mL/min, 260 nm
A H_2O/0.05% TFA
B 50% IPA/MeOH/0.05% TFA
80-100%B over 10 minutes, 100%B for 15 minutes
Retention time: HAA1-36OH, 23.3 minutes.
Retention time: AcAA1-$36NH_2$, 22.7 minutes.

3) The product and starting material do not separate well on TLC or reverse phase HPLC.

9.5 Preparation of T-20 Side by Side-chain deprotection of AcAA1-$36NH_2$

The Example presented in this Section demonstrates the successful coupling of solid and liquid phase synthesis techniques to produce a T-20 peptide from peptide intermediate fragments. In particular, the synthesis route described here represents the T-20 three fragment approach shown in FIG. 4.

Structure:

Ac-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-LeuGlu-Leu-Asp-Lys-Trp-Ala-Ser-Leu-Trp-Asn-Trp-Phe-NH_2 (SEQ ID NO:1)

$C_{414}H_{543}N_{51}O_{74}$
MW 4492.1

Materials:	MW	eq	mmoles	grams	mL
$AcAA1\text{-}36NH_2$	7411.95	1	0.035	0.26	
Trifluoroacetic acid					2.25
Water					15.1
Dithiothreitol				0.12	
MTBE					170
Acetonitrile					15

Theoretical Yield 157mg **Expected Yield:** 25-50%

Procedure:

A solution of 90:5:5 (v/v/wt %) trifluoroacetic acid/water/dithiothreitol was degassed with nitrogen and cooled to 0-5°C. To the cooled solution was added $AcAA1\text{-}36NH_2$ (as synthesized in Section 9.4, above). The slurry was stirred at 0-5°C until the solids dissolved (~ 5 minutes) then warmed to room temperature and stirred for 2.5 hours. The solution was added to 0-5°C MTBE (70 mL) to precipitate the peptide. The slurry was spun in a centrifuge for five minutes at 2200 rpm and the MTBE decanted from the solids. The solids were again suspended in MTBE (50 mL), spun in a centrifuge for five minutes at rpm and the MTBE was decanted. This process was repeated once more then the solids were dissolved in 1:1 water/acetonitrile (30 mL) containing 1 vol% acetic acid and stored at room temperature for 24 hours (note 1). The solution was frozen then freeze dried using a lyopholyzer to give 155 mg of crude T-20. Purification by preparative HPLC provides 55 mg of the full-length T-20 peptide in 95A% purity by HPLC (note 2).

Notes:

1) The tBu side-chain of Trp(Boc) is removed quickly leaving TrpCOOH. Decarboxylation of the TrpCOOH requires a minimum of 24 hours at ambient temperature in aqueous acetic acid.

2) Preparative HPLC
2" YMC, 120A, 10μ, C18
220 nm, 50 mL/min
A H_2O/0-1 % TFA
B ACN/0. 1 % TFA
39-49% B/40 minutes

10. EXAMPLE: PURIFICATION OF T-20 PEPTIDE

The Example presented herein describes methods by which T-20 and T-20-like peptides can be purified under conditions which greatly increase peptide purification throughout.

MATERIALS

Column used: 20 x 30 cm packed with Amberchrom CG-300S (Tosohaus; Montgomeryville, PA) 35μm particles.

PREPARATION OF BUFFERS

Buffer A = 100 mM Ammonium acetate adjusted to pH 8.5 with NH_4OH.

Buffer B = acetonitrile.

1. Column with approximately 6 column volumes of 20% B.
2. T-20 is dissolved in 50-100 mL of 85%A/15%B per gram of peptide. The pH is adjusted to 8-10 with 2M K_2CO_3. The acetonitrile concentration in the T-20 sample does not exceed 15-20%.
3. T-20 solution is loaded at 500 mL/min, with column pressure monitoring.
4. After T-20 solution is loaded, a 3 column volume (10 L) of a solution of 80%A/15%B is loaded to wash out lines.

5. Column eluate is monitored at 303 mn, and eluate is collected during entire loading process. The wavelength or attenuation is adjusted during the run to keep peak on scale. Absorbance is a function of wavelength and cell path length.
6. Gradient operation is begun as below (1.6% change in B/hour), with pressure being monitored during entire run.

Time (min)	%B	Flow (mL/min)
0	15	330
788	36	330

7. 10 min fractions are collected (3.3L) when main peak begins to elute. All of T-20 should be eluted by 35%B. On average, 35-40 fractions are collected. Fractions are stored at 0-5° C until a determination of which fraction to pool is made.
8. Fractions are collected until detector absorbance is less than 0.1 AU or until a major inflection point is reached after the main peak elutes.
9. The purity of each fraction is monitored by analytical reversed-phased HPLC.

RESULTS

T-20 peptide is much more soluble at pH ranges greater than 7. Column supports commonly used to purify peptides are silica-based and can, therefore, only be used at pH ranges because silica support tends to dissolve at higher pH ranges.

The method described herein utilizes a polystyrene-based resin support that is stable at a broad pH range (pH 1-14). This method greatly increases the capacity of the column in that T-20 throughput increased from 10g up to 250-450g (for an 8" diameter x 30 cm column).

11. EXAMPLE: LARGE SCALE SYNTHESIS AND PURIFICATION OF T-20 PEPTIDE RESIN LOADING

FmocTrp(Boc)OH can be loaded on very active 2-CTC resin at levels of 1.2 mmole./g of starting resin or higher. At levels over 1.1 mmole of FmocTrp(Boc)OH per gram of starting resin (over 0.72 mmol/g on loaded resin), it is difficult to obtain negative ninhydrin tests for the last three amino acids of the fragment. Likewise, it becomes difficult to build FmocAA17-26O-resin when the FmocLeuO-resin is loaded greater than 0.85 mmole/g and AcAA1-16O-resin when FmocGlnOH-resin is loaded greater then 0.75 mmole/g.

Upon receipt of the resin from the vendor, a use test with about 1 g of 2-CTC resin is conducted with each amino acid to be loaded. The purpose of the use test is to determine the quantity of amino acid to be used during loading to obtain the desired loading range. Use 0.8, 1.0 and 1.5 equivalents of FmocGlnOH, 1.0, 1.2 and 1.5 equivalents of FmocTrp(Boc)OH and 0.8, 1.0 and 1.5 equivalents of FmocLeuOH with 0.5 equivalent excess of DIEA for each (relative to the reported activity of the resin). If 1 mole of FmocLeuOH cannot be loaded onto 1 Kg of 2-CTC resin, the 2-CTC should not be accepted from the vendor. Below is a description of the targeted resin load for FmocLeuOH, FmocGlnOH and FmocTrp(Boc)OH.

From 1.0 to 1.1 moles of FmocLeuOH can be loaded onto each Kg of 2-CTC resin. Taking into account loss of HCl, the dry weight of the resin after loading FmocLeuOH should be 1.32 to 1.35 times the weight of starting resin and the measured loading should be 0.75 to 0.81 mmole/g.

From 0.8 to 1.0 moles of FmocGlnOH can be loaded onto each Kg of 2-CTC resin. Taking into account loss of HCL, the dry weight of the resin after loading FmocGlnOH should be 1.27 to 1.33 times the weight of starting resin and the measured loading should be 0.63 to 0.75.

From 0.9 to 1.1 moles of FmocTrp(Boc)OH can be loaded onto each Kg of 2-CTC resin. Taking into account loss of HCL, the dry weight of the resin after loading FmocTrp(Boc)OH

should be 1.44 to 1.54 times the weight of starting resin and the measured loading should be 0.63 to 0.72.

For accurate weight gain analysis, the loss on drying (LOD) of the starting resin must be determined. It should not exceed 1%. If residual solvent (or DIEA/HCl) is not completely removed from the resin during drying (after the loading), then the isolated mass will be higher and the measured loading lower. However, the total moles loaded (mass times measured loading) should fall within the above mentioned ranges. If the high-end loading level is exceeded (or total moles loaded per Kg of starting resin) for any of the amino acids mentioned, by over 10%, run a use test using less amino acid. This is particularly important for FmocGlnOH.

11.1 Large Scale Preparation of Fmoc-AA(Boc)-2-Chlorotrityl Resin

The purpose of the following section is to perform a large scale loading of resins suitable for preparation of high quantities of peptides for solid phase peptide synthesis (SPPS). The resin is charged with each of the following: FmocTrp(Boc)OH, and FmocGln(Boc)OH, and FmocLeu(Boc)OH. Each starting residue was charged onto approximately 4 Kg of 2-CTC resin.

AA Loaded	2-CTC (Kg)	Loaded Mass (Kg)	Substitution (mmol/g)[1]	Total Moles Loaded[1]
FmocTrp(Boc)OH	3.7	4.493	0.56 (0.56)	2.52 (2.52)
FmocLeu(Boc)OH	2.2	2.436	1.07 (0.647)	2.6 (1.58)
FmocGln(Boc)OH	3.65	3.856	0.55 (0.52)	2.12 (2.0)

1. The first number was calculated from a weight based HPLC assay against an Fmoc-AA standard. The value in parentheses was calculated from weight gain taking into account a 12% LOD on the starting 2-CTC resin.

Starting 2-CTC resin was purchased from Colorado BioTech with a reported loading of 1.4 meq/g. The standard loading procedure, 1.5 eq of FmocTrp(Boc)OH, 1.7 eq of DIEA in DCM (5

vol), ambient temperature for 2 hours, was used starting with 3.7 Kg of 2-CTC. This provided 4.493 Kg of FmocTrp(Boc)O-resin that had a loading calculated at 0.56 mmol/g (2.52 moles total) by weight based HPLC assay of FmocTrp(Boc)OH after cleavage from the resin. By weight gain of the resin, the loading is calculated to be 0.36 mmol/g for a total of 1.62 moles prepared. However, if the 12% LOD on the starting 2-CTC resin is taken into account, the loading calculated by weight gain is the same at 0.56 mmole/g for a total of 2.52 moles, well short of the targeted loading of 4 moles FmocTrp(Boc)OH per 4 Kg of resin.

A total of 4.59 Kg of FmocLeuO-resin was prepared from 3.9 Kg of 2-CTC in two runs using standard loading procedures.

However, a substantially lower loading than was anticipated was obtained. The first batch had a calculated loading of 1.07 mmol/g by weight based HPLC analysis of FmocLeuOH after clevage from the resin. This is clearly impossible as the loading calculated by weight gain taking into account the 12% LOD on the starting 2-CTC resin, is (500 g) 0.647 mmole/g. The actual loading is likely close to 0.647 mmole/g for a total of 1.577 moles loaded.

A second batch of FmocLeu-O-resin prepared (2.154 Kg) from 1.7 Kg of 2-CTC had a calculated loading of 0.68 mmole/g versus a weight gain loading of 0.66 mmole/g.

A total of 3.856 Kg of FmocGlnO-resin was prepared in a single batch starting with 3.65 Kg of 2-CTC using 0.8 eq of FmocGlnOH and 1 eq of DIEA in 7 volumes of 5/2 DMF/DCM. The substitution calculated by weight based HPLC assay was 0.55 mmol/g for a total of 2.12 moles of FmocGlnO-resin. The loading calculated by weight gain taking into account the 12% LOD of the starting 2-CTC resin is 0.52 mmol/g for a total of 2.0 moles FmocGlnO-resin.

Generally, the loading by weight gain should be greater than or equal to the loading calculated by a weight based HPLC assay against the AA loaded or fulvene removed. The

starting 2-CTC should have less than 1% LOD and the isolated FmocAAOresin will likely have a small amount of NMP, salt and/or residual FmocAAOH.

11.1.1 Preferred Method for Large Scale Preparation of Fmoc-AA(Boc)-2-Chlorotrityl Resin

FmocTrp(Boc)OH and FmocLeuOH

The air sensitive 2-Chlorotritylchloride resin (1 eq, 3.7 Kg, 5.18 mole) is added to a SPPS chamber and washed with 5 volumes of DCM. The solvent is drained and a solution of either FmocTrp(Boc)OH or FmocLeu(Boc)OH (1.5 eq) and DIEA (1.7 eq) in DCM (5 vol) is added (note 1). The slurry is agitated with stirring for 2 hours. The solvent is drained and the remaining active sites on the resin are end-capped with 9:1 MeOH:DIEA (5 vol) for 30 minutes. The solvent is drained and the resin is washed with 6 x 5 volumes of DCM. The resin is dried to constant weight, then sampled and analyzed for loading (note 2). The same procedure is used for loading FmocLeuOH.

FmocGluOH

The air-sensitive 2-CTC (3.65 Kg, 1.4 mmole/g) resin is charged into a 40L SPPS chamber under an atmosphere of nitrogen and washed with DCM (5 vol). A 20L reactor is charged with FmocGlnOH (1.506 Kg, 0.8 eq), DMF (5 vol) and DIEA (1.0 eq). The SPPS chamber is drained and the DMF solution is added. The reactor is rinsed with DCM (2 vol) and the rinse added to the SPPS chamber. The resin is stirred in the SPPS chamber under a nitrogen atmosphere for 2 hours and drained. Any remaining active sites on the resin are capped with 9:1 Methanol:DIEA (5 vol) for 30 minutes. The resin is drained, washed with DCM (6 x 5 vol) and dried to constant weight (3.856 Kg). The resin is sampled and tested for loading level (note 2).

Notes:

1. The 2-Chlorotritylchloride resin is very sensitive to moister. Water caps the resin and yields HCl. As long as the solvent is dry, there is little difference between DCE and DCM.
2. Resin loading is determined by cleaving the Fmoc amino acid from the resin and assaying against a standard with a quantitative wt/wt HPLC analysis.
   Phenomenex Jupiter C18, 300A, Sm
   1 mL/min, 260 nm
   A: 0.1% aqueous TFA
   B: 0.1% TFA in acetonitrile
   65%B, isocratic
   Retention time: approximately 8 minutes

Determination of the load by weight gain of the resin can be inaccurate as the number of washes used prior to drying the resin may not be sufficient to completely remove NMP from the resin. The calculation is:

(<u>Mass Fmoc-AA-OH-resin - Mass of stating 2-CTC</u>)/Mass Fmoc-AA-OH-resin (MW of AA loaded - MW HCl)

It was observed that storing the peptide fragments of the present invention for prolonged periods of time (days) in DCM results in substantial cleavage from the resin. For example, during the course of building FmocAA17-26OH, the partially built fragment was stored over the weekend twice in DCM. An 8% yield of FmocAA17-26OH was obtained after completion of the synthesis and removal from the resin. It is suspected that trace HCl in the DCM slowly cleaved the partially built fragment from the resin during the weekend storage.

Samples of FmocAA17-26O-resin, AcAA1-16O-resin and FrmoAA27-35O-resin were allowed to stand in DCM and IPA at room temperature for 1 week. The supernatant for each was analyzed by HPLC. Although quantitative results were not

obtained, all the fragments were cleaved from the resin to a significant extent in DCM while cleavage in IPA was not observed. If partially built fragments need to be stored for a few days (over the weekend), it is better to rinse the solids with NMP, drain the bed and let it stand saturated with NMP under a nitrogen atmosphere.

DCM was replaced with IPA for the final washes of FmocLeuO-resin, FmocGlnO-resin, FmocTrp(Boc)O-resin, FmocAA17-26O-resin, AcAA1-16O-resin and FrmoAA27-35O-resin. The IPA saturated FmocLeuO-resin, FmocGlnO-resin and FmocTrp(Boc)O-resin were dried at 40°C. FmocAA17-26O-resin, AcAA1-16O-resin, and FrmoAA27-35O-resin were built from the loaded resins that were dried at 4°C. At completion of the synthesis, the resin bound fragments were washed clean with DOM followed by IPA. The IPA saturated, resin bound fragments were divided and dried at ambient temperature and 40°C. The fragments were cleaved from the resin and analyzed by HPLC. There were no observed differences in the fragment purity. Drying IPA saturated, resin bound fragments at 40°C does not impact the quality or quantity of the fragment isolated.

SPPS of the fragments in DMF as opposed to NMP/DCM was examined (1 g of resin swells to 5 mL in both solvent systems). Fragment FmocAA27-35OH was synthesized using DMF as a solvent and TBTU vs HBTU as a coupling reagent. FmocAA27-35OH was isolated in 77% yield and 89.5A% purity.

The work up protocol for fragments FmocAA17-36NH_2 and AcAA1-36NH_2 removes the uncoupled fragments. Acetylating (endcapping) these fragments at deletion sites during the solid phase synthesis will ensure that the deletion fragments do not couple in the solution phase couplings. The uncoupled fragments in the synthesis of both FmocAA17-36NH_2 and AcAA1-36NH2 should be removed in the IPA and ACN work ups.

11.2 Large Scale Preparation of Fragment Ac-AA(1-16)-OH(Fragment 3c)

Large scale preparation by SPPS of fragment AcAA1-16OH was conducted in two runs yielding a total of 7.941 Kg of resin bound fragment from 3.53 Kg of FmocGlnOH-resin. The loading value for the starting FmocGlnOH-resin in both runs was the somewhat overestimated 0.55 mmol/g value obtained from a weight based HPLC assay rather then the more accurate 0.52 mmol/g obtained by weight gain taking into account 12% LOD on the starting 2-CTC. In addition to having the resin loading lower then desired, a 6% excess of each amino acid was used during the SPPS.

The SPPS used 2.5 equivalents of the first FmocGln(trt)OH onto the resin, and 1.7 equivalent of each of the subsequent amino acids in the fragment (relative to the 0.55 mmol/g loading). The coupling reactions were conducted in 8 volumes of 3:1 NMP:DCM and all the but two of the 32 coupling reactions (acetylation included) went to completion within 2 hours. All washes were done with 5 volumes of NMP.

The resin-bound fragment is washed with 5 to 6 times in 3.4 volumes (relative to the weight of the dry AcAA1-16O-resin) of 1% TFA/DCM at 0-5°C, 5 minutes per wash. Each wash is collected onto 1.36 volumes of 0.33 M aq $NaHCO_3$ to neutralize the TFA. The triphasic washes are combined and water (2 vol) is added. The DCM is removed by distillation leaving a filterable precipitate in the aqueous sodium bicarbonate. During the course of the distillation, the multi-phasic slurry becomes viscous and cream-like before collapsing into a filterable slurry as the last of the DCM is removed. The slurry is cooled to 0-5 °C and the pH is adjusted to 3.0 with 0.1 N HCl. The slurry was stirred at 0-5 °C for 1-2 hours, collected by filtration, washed and dried. In the remaining runs the solids were collected, returned to the reactor and triturated with water for 1-2 hour, then collected and dried.

Cleavage of AcAA1-16OH from the resin was accomplished in four runs using 1% TFA in DCM. A total of 4.814 Kg of AcAA1-16OH was produced from 3.53 Kg of FmocGlnO-resin in 93-96A% purity. The overall yield based on the 0.52 mmol/g loading determined by weight gain taking into account the 12% LOD of the starting 2-CTC (5.83 Kg theoretical) is 83%.

The sodium salt of AcAA1-16OH is insoluble in DMF and NMP, therefore, the pH adjustment is needed to protonate the carboxyl terminus. The water wash ensures removal of the sodium trifluoroacetate from the product. A very small amount of sodium trifluoroacetate on a wt/wt basis will interfere with the solution-phase coupling with $HAA17\text{-}36NH_2$.

This fragment should be dried at ambient temperature. A stability study looking at drying wet AcAA1-16OH at 40 °C showed about 4% degradation over 3 days. Interestingly, the dry solid is stable at 80°C for 24 hours.

11.2.1 Preferred Method of Large Scale Preparation of Fragment Ac-AA(1-16)-OH(Fragment 3c)

To a 40 L peptide chamber is added the resin-bound FmocGlnOH (1.53 Kg, 0.55 mmol/g, 0.84 mole). The resin is conditioned with DCM (5 vol) under nitrogen with agitation for 15 minutes, then drained. A 20% piperidine in NMP solution (5 vol) is added and the suspension is stirred under nitrogen for 20 minutes. The solution is drained and the process repeated. The resin is washed 5 times with 5 volumes of NMP to remove dibenzofulvene and piperidine as determined by a chloranil test (note 1).

While the resin is being washed clean of piperidine, the subsequent amino acid in the sequence (1.5 eq), HOBT (1.5 eq) and DIEA (1.5 eq) are combined in NMP (6-7 vol) and cooled to 0°C. To the cool solution is added HBTU (1.5 eq) and the solution is stirred for 10-15 minutes to dissolve the HBTU. The cooled solution of activated amino acid is added to the resin followed by a DCM rinse (2.5 vol) (note 2). The suspension is stirred under nitrogen purge for 2 hours, then

a sample of the resin is removed for a qualitative ninhydrin test (note 3). If the ninhydrin test is negative, the vessel is drained, washed with 3 times 5 volumes of NMP (note 4) and the cycle is repeated with the next amino acid in the sequence. If the test is positive, the suspension is agitated for an additional hour and retested. If the ninhydrin is negative, proceed to the next cycle. If the ninhydrin remains positive, recouple with one eq of the amino acid and reagents. If the ninhydrin is positive after one hour, endcap with Seq of acetic anhydride and Seq of pyridine in NMP (10 vol) for one hour.

At completion of the fragment synthesis, the Fmoc is removed from the last amino acid as described and the resin is stirred in a solution of acetic anhydride and pyridine (5 eq each) in 3:1 NMP:DCM (10 vol) for 20-30 minutes or until a negative ninhydrin test is obtained. The resin is drained, washed with 2 X 5 volumes of NMP, 5 times with 5 volumes of DCM and dried yielding 3.49 Kg of AcAA1-16O-resin.

The 40 L SPPS chamber is charged with the dried, resin-bound AcAA1-16 (3.49 Kg). The resin-bound peptide is cleaved from the resin using 6 X 3.4 volumes of 1% TFA/DCM (note 7). Each cleavage wash is collected onto 1.36 volumes of 0.33 M aq. sodium bicarbonate. The biphasic fractions are combined and diluted with 2 volumes of water. The biphasic mixture is concentrated under reduced pressure (15 in Hg, 20 °C) to remove DCM. Additional water (2 volumes) is added during the distillation as needed to maintain stirring (note 6). When the DCM is removed (several hours), the suspension is cooled to 0 °C and the pH is adjusted to 3 with IN aq HCl. The slurry is stirred at 0 °C for 1 hr and collected. The still damp solid is returned to the reaction vessel and triturated with water (7 volumes) to remove residual TFA and sodium trifluoroacetate. The solids are collected by vacuum filtration and dried to a constant weight (1.12 Kg, 95A%). The yield AcAA1-16OH based on 0.52 mmol/g load is 86% (note 7).

Notes:

1. To approximately 1 mL of acetone is added 1 drop of a saturated solution if chloranil in toluene, followed by one drop of effluent. A blue or violet color is a positive indication for the presence of a piperidine. As the bed height increases, larger volumes of NMP will be needed to wash out the piperidine.
2. DCM is added to ensure adequate swelling of the resin.
3. Quantitative ninhydrin testing is adequate for monitoring the coupling efficiency. A 2-20 mg sample of the resin is withdrawn and washed clean with methanol. To the sample is added 3 drops of 76% phenol in ethanol, 4 drops of 0.2 mM KCN in pyridine and 3 drops of 0.28 M ninhydrin in ethanol. The solution is diluted to 0.5-1 mL with ethanol and placed in a heat block at 75°C for 5-10 minutes. A blue or violet color indicates free amines (positive). Clear or faint blue is a negative result. Protocol for Quantitative Ninhydrin Test References: Sarin, V. K., Kent, S. B. H., Tam, J. P., & Merrifield, R. B. (1981) Analytical Biochem. iii, 147-157.
4. If the resin is to be stored overnight, wash with 2 times 5 volumes of NMP, drain and store under nitrogen. Do not store under DCM. This can result in cleavage of the peptide from the resin and substantial mass loss.
5. Each fraction is tested for product content by TLC visualization at 254 nm. The majority of the product is removed in the first 5 washes.
6. As the DCM is removed, the reaction takes on the consistency of marshmallow cream. As the distillation continues, the suspension gradually breaks into a solid slurry. It is important to remove the DCM slowly to avoid product oil-out.
7. Vydac C8, 5 μ, 300A
   1 mL/min, 30°C, 230 nm
   A 1000:1 water/TFA

B 800:200:1 IPA:ACN:TFA
60-95%B/30 min.
Retention time: 13.1 minutes

11.3 Large Scale Preparation of Fragment FmocAA(17-26)-OH(Fragment 10b)

A total of 5.3 Kg of FmocAA17-26O-resin was prepared from 2.4 Kg of FmocLeuO-resin in two, equal size runs. The 5.3 Kg of FmocLeuO-resin was cleaved from the resin in four equal size runs yielding 3.184 Kg of FmocAA17-26OH averaging 94.4A% purity and 90% yield.

The reactions were run using 1.5 equivalent of each amino acid relative to the loading factor, resulting in the use of 65% excess of amino acid for each coupling cycle. All of the coupling reactions were complete (negative ninhydrin test) within 2 hours.

The Fmoc protecting group was removed with 20% piperidine in NMP (5 volumes) for 20 minutes and repeated. The piperidine was washed out of the resin with 5 x 5 volume NMP washes. The coupling reactions were run in 8 volumes of 3:1 NMP:DCM. The coupling solution was drained and the solids were cleaned with three, 5 volume NMP washes. The cycle was repeated with the next amino acid in the sequence. At completion of the fragment, the resin bed was washed with 2 x 5 volumes of NMP, 5 x 5 volumes of DCM and dried to constant weight.

Several 1% TFA in DCM washes are used to remove the fragment from the resin. Cooling the 1% TFA in DCM solution is not necessary as the stability of this fragment in 1% TFA/DCM (1%/day) is much greater then AcAA1-16OH. As the product containing, acidic DCM washes are collected, they are neutralized with pyridine. The combined washes are distilled to remove DCM and ethanol is added to maintain a solution as well as to drive the remaining DCM out during the distillation. After the DCM has been removed (determined by an increase in the temperature of the distillate being removed) water was added to precipitate the fragment. The

solid was collected by vacuum filtration (less than 60 minutes). The still damp solid was transferred back to the reactor and triturated with 80/20 ethanol/water (5 vol) at 0-5 °C for 60 minutes. The precipitated FmocAA17-26OH was collected by vacuum filtration, ashed with a minimal amount of 80/20 ethanol/water and dried (vacuum, no heat, 10 days).

11.3.1 Preferred Method for Large Scale Preparation of Fragment FmocAA(17-26)-OH(Fragment 10b)

A 40L SPPS chamber is charged with FmocLeuO-resin (1.2 Kg, 0.776 mole) followed by DCM (5 vol) to swell the resin. The DCM is needed to ensure complete swelling of the dried starting resin. The suspension is stirred for 20-30 minutes and drained. A 20% piperidine in NMP solution is added (5 vol) and the solution is stirred for 20 minutes. The solvent is drained and the process is repeated. The solvent is drained and the resin bed is washed with 5 x 5 volumes of NMP to remove piperidine (note 1).

While deprotecting, a 20 L round bottom flask with mechanical stirred is charged with the next amino acid in the sequence (1.95 eq), HOBT (1.95 eq), DIEA (1.95 eq) and NMP (6 vol). The solution is stirred until the solids dissolve, then cooled to 0-5 °C and HBTU (1.95 eq) is added. The solution is stirred until the HBTU dissolves or the resin if free of piperidine (whichever is first), then added to the resin. The reactor is washed with DCM (2 vol) and this is transferred to the SPPS reactor. Note: The stoichiometry of amino acid and reagents should be 1.5 equivalents.

The resin is suspended in coupling solution with gentle stirring. After two hours, a sample of resin is removed from the SPPS chamber and a qualitative ninhydrin test is performed (note 2). If the ninhydrin test is negative, the vessel is drained, washed with 3 times 5 volumes of NMP and the cycle is repeated with the next amino acid in the sequence (the DCM swelling is used only for the first amino acid loaded).

If the test is positive, the suspension is agitated for an additional hour and retested. If the ninhydrin is negative, proceed to the next cycle. If the ninhydrin remains positive, recouple with one eq of the amino acid and reagents. If the ninhydrin is positive after one hour, endcap with 5 eq of acetic anhydride and 5 eq of pyridine in NMP (10 vol) for one hour.

At completion of the fragment synthesis, the resin is drained, washed with 2 X 5 volumes of NMP, 5 X 5 volumes of DCM and dried yielding 2.67 Kg of FmocAA17-26O-resin.

FmocAA17-26OH is cleaved from the resin (1.33 Kg) using 5-6 x 1.7 volumes of 1% TFA in DCM, 5 minutes each. The 1% TFA/DCM washes are collected in a flask containing pyridine (1:1 volume ratio with the TFA in the wash). The product containing washes are combined (approximately 14 L) and the DCM is removed by distillation to a minimum pot volume (approximately one third of the original volume). The vacuum is adjusted to maintain a pot temperature of 15-25 °C. Ethanol (6.5 vol) is added and the distillation is continued until the DCM is removed (as determined by the temperature of the distillate, note 3). Again the vacuum is adjusted to maintain a pot temperature of 15-20 °C. The final pot volume should be approximately 8-10 volumes. The solution is cooled to 5-10°C and water (6.5 vol) is added over 30 minutes to precipitate the FmocAA17-26OH. The solid is collected by vacuum filtration and washed with water (2-3 vol). To remove residual pyridine and/or salts, the still damp solid is charged back to the reactor and 80/20 ethanol/water (5 vol), precooled to 0°C, is added. The suspension is stirred at 0°C for 60 minutes, the solids are collected by vacuum filtration, washed with a minimal amount of 80/20 ethanol/water and dried to constant weight to give 0.806 Kg of FmocAA17-26OH in 90.6% yield and 96A% purity (note 4).

Notes:

1 To 1 mL of acetone is added 1 drop of a saturated solution of chloranil in toluene, followed by 1 drop of effluent. A blue or violet color indicates the presence of piperidine.

2 Quantitative ninhydrin testing is adequate for monitoring the coupling efficiency. A 2-20 mg sample of the resin is withdrawn and washed clean with methanol. To the sample is added 3 drops of 76% phenol in ethanol, 4 drops of 0.2 mM KCN in pyridine and 3 drops of 0.28 M ninhydrin in ethanol. The solution is diluted to 0.5-1 mL with ethanol and placed in a heat block at 75°C for 5-10 minutes. A blue or violet color indicates free amines (positive). Clear or faint blue is a negative result.

3 The head temperature during the vacuum distillation of DCM was 10-15°C. The head temperature rose to 3500 when the DCM was removed.

4 Vydac, C8, 5μ, 300A
1 mL/min, 262 nm, 30°C
A water/0.1% TFA
B ACN/0.1% TFA
80-99%B/20 min.
retention time: 15.2 minutes

11.4 Large Scale Preparation of Fragment Fmoc-AA(27-35)-OH(Fragment 16b)

A total of 4.694 Kg of FmocAA27-35OH was synthesized from 4.45 Kg of FmocTrp(Boc)O-resin. The solid phase synthesis was run in two batches and cleavage from the resin was four batches. FmocAA27-35OH resulting from one batch was about 5% less pure than material from the other batch. This was due to an unidentified, 5A% impurity that was removed in processing to $FmocAA17\text{-}36NH_2$. The total amount of FmocTrp(Boc)OH loaded onto the resin was 2.5 moles. The solid phase synthesis proceeded as expected for resin loaded at about 63% of capacity. All of the couplings were complete

at the first, 2 hour check point. The reaction and rinse volumes used for the SPPS and cleavage were the same as those used in the synthesis of FmocAA17-26OH. Cleavage from the resin was as described above. Filtration was quick (15 min). Drying the solid after the 90/10 ethanol/water trituration took 3 days (vacuum, no heat). The fragment is stable to drying at 40 °C.

11.4.1 Preferred Method of Large Scale Preparation of Fragment Ac-AA(27-35)-OH(Fragment 16b)

To a SPPS chamber containing FmocTrp(Boc)-resin (1 eq, 2.2 Kg, 1.23 moles) is charged DCM (5 vol). The suspension is stirred for 15 minutes and drained. A solution of 20% piperidine in NMP (5 volumes) is added and the suspension stirred for 10-15 minutes to remove the Fmoc protecting group. This process is repeated and the resin is washed with 5-7 x NMP (5 vol) to a negative chloranil test (note 1).

The subsequent amino acid (1.5 eq), HOBT (1.5 eq) and DIEA (1.7 eq) are combined in NMP (6 vol), then cooled to 0-5°C (note 2). HBTU is added and the solution is stirred for 10-15 minutes to dissolve. The solution of activated amino acid is added to the resin. DCM (2 vol) is used to wash the reactor then added to the resin (note 3). The suspension is stirred under an atmosphere of nitrogen for 1-2 hours. Coupling completion is monitored with a qualitative ninhydrin test (note 4). If the ninhydrin test is negative, the vessel is drained, washed with 3 times 5 vol of NMP and the cycle is repeated with the next amino acid in the sequence (the DCM swelling is used only for the first amino acid loaded).

If the test is positive, the suspension is agitated for an additional hour and retested. If the ninhydrin is negative, proceed to the next cycle. If the ninhydrin remains positive, recouple with one eq of the amino acid and reagents. If the ninhydrin is positive after one hour, endcap with 5 eq of acetic anhydride and 5 eq of pyridine in NMP (10 vol) for one hour.

At completion of the fragment synthesis, the resin is drained, washed with 2 X 5 volumes of NMP, 5 X 5 volumes of DCM and dried yielding 4.11 Kg of FmocAA27-350-resin.

The fragment is cleaved from the resin (2.05 Kg) using 6 x 1.7 volumes of 1% TFA in DCM, 5 minutes each. The 1% TFA/DCM washes are collected in a flask containing pyridine (1:1 volume ratio with the TFA in the wash). The product containing washes are combined and the DCM is removed by distillation (vacuum adjusted to maintain a pot temperature of approximately 15 °C) to approximately one half of the original pot volume. Ethanol (5 vol) is gradually introduced and the distillation (vacuum adjusted to maintain a pot temperature of approximately 20 °C) is continued until the DCM is removed (as determined by the temperature of the distillate, note 5). The pot volume should be 6-7 volumes. The cloudy solution is cooled to 10-15 °C and water (3.5 vol) is added over 30 minutes with rapid agitation to precipitate the FmocAA27-35OH. The solids are collected by vacuum filtration (15 minutes), and washed with water (1 vol). To remove residual pyridine, the damp solid is returned to the reactor and 90/10 ethanol/water precooled to 0-5 °C is added (10 vol). The slurry is stirred at 0-5 °C for 60 minutes, the solid is collected by vacuum filtration, washed with 90/10 ethanol/water (0.5 vol) and dried to constant weight giving 1.19 Kg of FmocAA27-35OH in 89% yield and 89.2A% purity (note 6).

This protocol can be reworked by triturating the solid with 15 volumes of 9:1 ethanol/water with stirring for 12 hours, followed by collection and drying.

Notes:

1. To 1 mL of acetone is added 1 drop of a saturated solution of chloranil in toluene, followed by I drop of effluent. A blue or violet color indicates the presence of piperidine.

2 The reagents less HBTU are added at room temperature to assist dissolution. HBTU is added to the cooled solution to minimize racemization.

3 Activation is carried out in NMP because HBTU is not soluble in DCM. DCM is used to wash the reactor and added to the resin to maintain adequate resin swelling.

4 Quantitative ninhydrin testing is adequate for monitoring the coupling efficiency. A 2-20 mg sample of the resin is withdrawn and washed clean with methanol. To the sample is added 3 drops of 76% phenol in ethanol, 4 drops of 0.2 mM KCN in pyridine and 3 drops of 0.28M ninhydrin in ethanol. The solution is diluted to 0.5-1 mL with ethanol and placed in a heat block at 75 °C for 5-10 minutes. A blue or violet color indicates free amines (positive). Clear or faint blue is a negative result.

5 The head temperature during the vacuum distillation of DCM was 10-15 °C. The head temperature rose to 35 °C when the DCM was removed.

6 Vydac, C8, 5 μ, 300A
1 mL/min, 262 nm, 30°C
A water/0.1% TFA
B ACN/0.1% TFA
80-99%B/20 min.
retention time: 15.3 minutes

11.5 Large Scale Preparation of Fragment FmocAA(27-36)-OH by Solution Phase Coupling of FmocAA(27-35)-OH with $HPheNH_2$

A total of 5.226 Kg of FmocAA27-$36NH_2$ was prepared in four runs from 4.676 Kg of FmocAA27-35OH. Residual coupling reagents or solvent account for the greater then 100% yield. These are removed in the next stage.

Solid sticking to the reactor wall after the water dropout was cited as a significant problem in this stage. Isolation of the crude solid by vacuum filtration took 30

minutes. The average drying time (vacuum, no heat) for the runs was 8 days. The solid needs to be compressed on the filter to remove excess water before it goes into the oven. The fragment is stable to drying at 40 °C and the vacuum ovens need to be heated.

A use test is run on a 0.5 to 1 gram scale with the batch/lot of starting materials to be used to help establish quality and identity. Thin Layer Chromatography (TCL) and HPLC are used to monitor conversion of starting material to product. The primary impurity to look for in fragment FmocAA27-35OH is trifluoroacetic acid (or a salt of it). Activation and reaction of trifluoroacetic acid present in the FmocAA27-35OH with the phenylalanine amide is a rapid process. A small excess of phenylalanine amide can be used to consume any trifluoroacetic acid present. A more significant quality issue is with the phenylalanine amide purchased. Most vendors sell this as the HCl salt. Several lots of the HCl salt of phenylalanine amide have produced an impurity (5-15A%) that co-elutes with the starting material on HPLC. The impurity can be separated from the starting fragment and product by TLC. The impurity is $FmocAA27\text{-}35NH_2$ resulting from ammonium chloride present in the HCl salt of phenylalanine amide.

11.5.1 Large Scale Preparation of Fragment FmocAA(27-36)-OH by Solution Phase <u>Coupling of FmocAA(27-35)-OH with $HPheNH_2$</u>

A 40L jacketed reactor with a mechanical stirrer is charged with FmocAA27-35OH (1 eq, 1.185 Kg), HOAT (1.1 eq), $HCl.HPheNH_2$ (1.15 eq) and DMF (12.5 vol). DIEA (2.1 eq) is added, the solution is cooled to 0-5 °C and HBTU (1.2 eq) is added. The reaction mixture is stirred for 15 minutes at 0-5 °C then warmed to room temperature and stirred an additional 70 minutes (note 1, HPLC used for in process check). After the reaction is judged complete by HPLC, water (12.5 vol) is added over 15-30 minutes to precipitate the peptide. The slurry is stirred for 15 minutes at ambient temperature. The

solids are collected by vacuum filtration, washed with water (3 vol) and dried to give $FmocAA27\text{-}36NH_2$ (1.357 Kg in 107% yield and 87.IA% purity by HPLC (note 2).

The reaction may be reworked by trituration of the solid with 15 volumes of 9:1 acetonitrile/water with stirring for 12 hours, collection and drying.

Notes:

1. In process control, TLC:
   88/12 dichloromethane/methanol
   UV, iodine detection
   Rf: FmocAA27-35OH, 0.49
   Rf: $FmocAA27\text{-}36NH_2$, 0.63
   Vydac C8, 5 m, 300A
   30°C, 1 mL/min, 262 nm
   A water/0.1% TFA
   B ACN/0.15 TFA
   80-99%B/20 minutes
   retention time: FmocAA27-35OH, 15.8
   retention time: $FmocAA27\text{-}36NH_2$, 17.12
2. A greater then theory yield is generally obtained unless the solid isolated is returned to the reactor and triturated with water.

11.6 Large Scale Preparation of Fragment HAA(27-36)-OH from FmocAA(27-36)-OH

A total of 3.897 Kg of $HAA27\text{-}36NH_2$ was prepared from 5.221 Kg of $FmocAA27\text{-}36NH_2$ in four runs with an average 2 step yield of 86.4%. The range in yield observed for the runs, 74-97%, presumably reflects carryover from one run into the next.

The work-up and product isolation in this stage works very well. The purity of the product is enhanced if the correct amount of DCM is left in the MTBE and the physical properties of the solid lead to rapid filtration and drying. This product isolation process has been incorporated into the new stage described in Section 11.6.2, below.

DCM is the solvent used during the deprotection of $FmocAA27\text{-}36NH_2$. At completion of the deprotection, the organic solution is washed with water twice to remove most of the excess piperidine, then concentrated to approximately one third of the original volume. MTBE is added to precipitate the fragment. The distillation is continued to remove the majority of the remaining DCM and complete the precipitation of the fragment. It is important to remove most of the DCM to avoid mass loss in the precipitation. It is also important to leave some DCM in the MTBE to ensure product cleanup. Dibenzofulvene, piperidine/fulvene adduct and residual piperidine are soluble in MTBE. The $HAA27\text{-}36NH_2$ is collected and dried. If necessary, a second trituration of the solid (12 hours) with MTBE will remove residual dibenzofulvene and more importantly the piperidine/fulvene adduct that may be present in the $HAA27\text{-}36NH_2$. A trituration with hexane will remove dibenzofulvene, but not the piperidine/fulvene adduct. Drying time of the solid isolated from MTBE is 1 day.

Due to the high solubility of $HAA27\text{-}36NH_2$ and related impurities in DCM/MTBE, an analytical method was desired to accurately determine the end point of the solvent exchange. A GC method has been developed to assay DCM in MTBE. The distillation is complete when the DCM content in the MTBE is below 6%.

11.6.1 Large Scale Preparation of Fragment <u>HAA(27-36)-OH from FmocAA(27-36)-OH</u>

A 40L glass jacketed reactor equipped with a mechanical stirrer and thermometer is charged with FmocAA27-36NH2 (1 eq., 1.356 Kg), DCM (5 vol) and piperidine (0.2 vol). The solution is stirred at ambient temperature for 1.5 hours (note 1). The organic solution is washed with water (2 x 5 vol). The volume of the organic layer is reduced to approximately one third the original volume by distillation (approximately 25 mm Hg, jacket temperature less than 45 °C to avoid melting the solid). MTBE (5 vol) is gradually

introduced into the reaction vessel while concentration continues to a point of heavy precipitation and the pot volume is approximately 5 volumes. The solvent exchange is stopped when the DCM content is below 6%. The slurry is cooled to 0-5 °C, stirred for 60 minutes then collected by vacuum filtration (rapid). The solids are washed with MTBE (2 x 0.5 vol) and dried to constant weight to give 1.022Kg of HAA27-36NH2 in 89.2% yield (2 steps) and 91.1A% purity (note 1).

The reaction may be reworked by trituration with MTBE (12.5 vol) at 23 °C for 13 hours, collection by vacuum filtration and drying.

Notes:

1. IPC and purity measured by HPLC:
   Vydac, C8, 5 , 300A
   1 mL/min, 262 nm, 30°C
   A water/0.1% TFA
   B ACN/0.1% TFA
   80-99%B/20 minutes
   retention time: FmocAA27-36NH_2, 16.5. HAA27-36NH_2, 8.4

11.6.2 Improved Preparation of Fragment HAA(27-36)-OH by Solution Phase Coupling of FmocAA(27-36)-OH with HPheNH_2

A new process that combines Sections 11.5 through 11.6.1, that takes FmocAA27-35OH to HAA27-36NH_2 directly has been developed. This new process removes issues associated with isolating FmocAA27-36NH_2 and a long drying time. A detailed description is given below. The new process removes a long drying time and a stage from the synthetic route.

Add FmocAA27-35OH (5.0g, I eq), HOAT (0.459, 1.2 eq) and Phe-NH_2 (0.389, 1.2 eq) to a 100 mL round bottom flask equipped with a magnetic stirrer and nitrogen inlet. Add 10 volumes of NMP (50 mL) and DIEA (0.45g, 1.5 eq) to the flask and stir under a nitrogen atmosphere until the solids dissolve. Cool the solution to 0 to 5 °C then add HBTU (1

.04g, 1.2 eq). Stir under a nitrogen atmosphere at 0 to 5 °C for 30 minutes. Warm the reaction mixture to 20 °C and continue stirring. Perform an in process check (IPC) 90 minutes after the HBTU was added (note 1).

After the in process check (IPC) shows the reaction is complete, add piperidine (1.379, 7 eq) to the reaction mixture and stir under a nitrogen atmosphere for 1.5 hours. Perform an IPC (note 1). If the Fmoc removal is not complete, stir an additional 30 minutes and run the IPC.

When complete, add the reaction mixture to 5% aqueous acetic acid at a rate as to not exceed a temperature of 35 °C (30 vol). Stir the resulting slurry for I to 2 hours and collect by vacuum filtration (note 2). Wash the solid with 10 volumes (50 mL) of water.

Return the damp solid to the reactor, add 20 volumes of water (100 mL) and stir at ambient temperature for 1 to 2 hours. Collect the solids by vacuum filtration, wash with 10 volumes of water (50 mL) and dry (note 3).

The dried (note 4) solid is triturated with MTBE (20 vol) at ambient temperature for 2 to 5 hours to remove dibenzofulvene and dibenzofulvene piperidine adduct. Collect the solid by vacuum filtration and dry to constant weight yielding 4.55 grams (94.3%) of HAA27-36NH_2 with 85-90A% purity (note 1).

Fmoc by products (dibenzofulvene and its piperidine adduct) are generally removed in this protocol, however, if a rework is necessary, stir the solid in 10 volumes of MTBE at 20°C for 2 to 3 hours, collect and dry.

<u>Notes:</u>

1. In process check (IPC) by Reverse Phase HPLC:

Column Vydac C8, 300A, 5µ, 30°C
Flow rate 1 mL/min
Detection UV at 262 nm
Mobile phase A. water/0.1 % TFA
B. acetonitrile/o.1% TFA
Method 80 to 99%B over 20 minutes
Retention Times:

FmocAA27-36NH_2 (16.5 min. HAA27-36NH_2 (8.4 min)

2. The total filtration time was 10 minutes..
3. The damp filter cake must be returned to the reactor for a water wash immediately to avoid oiling or gumming of the solids.
4. The crude product must dried completely to ensure that the MTBE trituration will remove the dibenzofulvene byproducts.

11.7 Large Scale Preparation of Fragment FmocAA(17-36)-OH by Solution Phase Synthesis fusion of FmocAA(17-26)-OH with HAA(27-36)-OH

A total of 5.115 Kg of FmocAA17-36NH_2 was prepared from 2.993 Kg of HAA27-36NH_2 and 3.176 Kg of FmocAA17-26OH in four runs. The average yield was 84.3%. Filtering the crude solid after the water dropout from the reaction mixture took an average of 40-50 minutes.

A use test is run on a 1-2 g scale testing both fragments and the HBTU to be used prior to running the coupling reaction. The stoichiometry of starting materials and reagents is determined from the use test. The solubility of the starting materials is also noted in the use test. A cloudy solution may indicate the presence of salts in FmocAA17-26OH and warrant a water wash on the fragment. It is imperative that all components of the reaction mixture are clearly in solution prior to addition of the HBTU to ensure complete conversion of the starting materials to product with minimal racemization and byproduct formation.

The purity of crude FmocAA17-36NH_2 isolated from the water dropout is significantly enhanced by precipitation from IPA. FmocAA17-36NH_2 is partially soluble is IPA. Trituration of FmocAA17-36NH_2 with approximately 15 volumes of 95/5 IPA/water pre-warmed to 60-70 °C followed by stirring while cooling to room temperature over several hours enhances the purity of the fragment. Both starting materials as well as the piperidine amide of FmocAA17-26OH and enamine urea adduct

of $HAA27\text{-}36NH_2$ are soluble in the 95% IPA. Trituration of the crude FmocAA17-36NH2 with 95% IPA at room temperature for extended periods of time is not as effective at removing impurities. A stability study at 60-70 °C has been completed. Failure to warm the IPA solution beyond 52 °C appears to have minimal impact on the quality of the product isolated.

11.7.1 Preferred Method for Improved Large Scale Preparation of Fragment FmocAA(17-36)-OH by Solution Phase Synthesis fusion of FmocAA(17-26)-OH with HAA(27-36)-OH

A 40L jacketed reactor fitted with a mechanical stirrer and nitrogen inlet is charged with FmocAA17-26OH (1 eq., 0.770 Kg), $HAA27\text{-}36NH_2$(1 eq., 0.720 Kg), HOAT (1.5 eq.) and DMF (12.5 volumes relative to FmocAA17-26OH). $EtPr_2N$ (1.5 eq.) is added and the suspension is stirred at room temperature until the solids are dissolved (visually). The solution is cooled to 0-5°C under an atmosphere of nitrogen and HBTU (1-1.05 eq) is added. The reaction mixture is stirred at 0-5 °C until the HBTU dissolves (visually, about 15 minutes). The reaction mixture is warmed to 25 °C and stirred for 2 hours (note 1). The DMF solution is cooled to 0-5 °C and cold process water (12.5 vol) is added at such a rate as to not exceed 25 °C. The resulting slurry is stirred for 1-24 hours, the solids are collected by vacuum filtration and washed with water (3 x 4 vol). The solids are dried on the filter for 16-24 hours to approximately 2 times the theoretical mass. The 40 L reactor is charged with 95/5 isopropanol/water (25 vol) and warmed to 45°C. The semi-dry $FmocAA17\text{-}36NH_2$ is added to the IPA solution with rapid agitation. The suspension is warmed to 52 °C, then stirred for 12-16 hours as it cools to room temperature (note 2). The solids are collected by vacuum filtration, washed with a minimal amount of IPA and dried to give 1.261 Kg of $FmocAA17\text{-}36NH_2$ in 85% yield and 90.SA% purity by HPLC (note 1). The

reaction may be reworked by repeating the 95/5 IPA/water trituration.

Notes:

1. In process control and purity, HPLC
   Vydac, C8, 5 μ, 300A
   1 mL/min, 262 nm, 30°C
   A water/0.1% TFA
   B 80/20 IPA/ACN/0.1% TFA
   60-95%B/20 minutes
   Retention times: $HAA27\text{-}36NH_2$, 6.73 minutes
   FmocAA17-26OH, 10.67 minutes
   $FmocAA17\text{-}36NH_2$, 20.2 minutes
2. The $FmocAA17\text{-}36NH_2$ will not completely dissolve in this volume at this temperature. Before collecting the solids, stop agitation and allow the solids to settle. Remove a sample of the filtrate and analyze by HPLC. If the filtrate contains a significant amount of FmocAA17-$36NH_2$, cool to 0°C before collecting the solids.

11.8 Large Scale Preparation of Fragment HAA(17-36)-OH from FmocAA(17-36)-OH

A total of 4.965 Kg of $HAA17\text{-}36NH_2$ was prepared from 5.112 Kg of $FmocAA17\text{-}36NH_2$ in four runs. Both the isolated yield and HPLC traces indicate the presence of dibenzofulvene and perhaps solvent. The dibenzofulvene present will not interfere with the next coupling reaction as it was removed with potassium carbonate, not piperidine, therefore, the piperidine adduct of dibenzofulvene is not an issue chemically.

There are several problems associated with this process. The reaction is run in 12 volumes of DMF. The base removing the Fmoc group is I volume of 1.1 M potassium carbonate, which is incapable of forming a difficult to remove, basic adduct with dibenzofulvene. The deprotection proceeds in about 90 minutes at room temperature.

A 1:1 saturated aqueous sodium chloride:water solution, precooled to 0 °C is added to co-precipitate the fragment and dibenzofulvene. This generates a fine, milky solid which takes hours (5-8) to filter. Once isolated, the damp solid has to be dried completely (less than 1% LOD) to remove the dibenzofulvene impurity. The drying takes 10 days (vacuum, no heat). Once dry, the solid is returned to the reactor and triturated with 3:1 heptane:MTBE (20 vol) at ambient temperature for 18 hours to remove the dibenzofulvene. Shorter trituration times do not remove it completely and if there is any water on the solids, it is not removed. A 3:1 heptane:MTBE (20 vol) rework can be used if dibenzofulvene persists.

To resolve these problems, the following new procedure was developed. Add FmocAA17-36NH_2 (2.0 g, 1 eq.) and heptane (8 vol) to a 100 mL round bottom flask equipped with a mechanical stirrer, temperature controller and reflux condenser. Add 2 volumes of MTBE (4 mL) and heat the slurry to 45-50°C (Note 1). Add the piperidine (1 0 eq.). Stir under a nitrogen atmosphere at 45-50°C for 24-36 hours (Note 2). Hot filter the reaction mixture at 45-50°C (Note 3), then wash the cake with 5 volumes (10 mL) of 60:40 heptane:MTBE.

__Notes:__

1. Slower reaction will result if MTBE is allowed to escape the reactor thereby changing the heptane:MTBE ratio. Reaction temperatures greater than 50°C can result in gumming of the reaction solids. The Fmoc is largely removed in 5 hours.
2. Reaction completion is monitored by RP-HPLC:

Column	Vydac C8, 300A, 5 , 30°C
Flow rate	1 mL/min
Detection	UV at 262 nm
Mobile phase	A. water/0.1% TFA
	B. 80:20 IPA:CAN 0.1 %TFA

Method 60 to 95%B over 30 minutes

3. Hot filtration aids in the removal of the piperidine adduct of dibenzofulvene.

11.8.1 Large Scale Preparation of Fragment <u>HAA(17-36)-OH from FmocAA(17-36)-OH</u>

A 40L jacketed reactor is charged with FmocAA17-36NH_2 (1 eq, 1.26 Kg) and DMF (12 vol) at room temperature. A 1.11 M aqueous potassium carbonate solution (1 vol, 5 eq) is added at once (note 1). The reaction mixture is stirred at room temperature for 3.5 hours (note 2). The reaction mixture is added slowly to a 1:1 solution of saturated aqueous sodium chloride/water (10 volumes) pre-cooled to 0 °C at such a rate as to not exceed a temperature of 10 °C (approximately 1-2 hr). The solid is collected by vacuum filtration using a rubber dam to compress water from the filter cake (note 3). The wet cake is transferred back to the reaction vessel and triturated (agitated) with water (10 vol) for 1-2 hours to remove residual inorganic salts. The solid is collected by vacuum filtration, compressed with a rubber dam then transferred to a vacuum oven and dried to constant weight. The dry solid (note 4) is triturated (agitated) with 3:1 heptane:MTBE (20 vol) for a minimum of 14 hours at room temperature to remove dibenzofulvene. The solid is collected by vacuum filtration, washed with heptane (2 vol) and dried to give 1.20 Kg HAA17-36NH2 in 99.5% yield and 75A% purity. The HAA17-36NH2 contains 5A% dibenzofulvene. Rework the reaction by repeating the heptane:MTBE trituration.

<u>Notes:</u>

1. The solution turns cloudy when the aqueous potassium carbonate is added, but clears with continued stirring. An exotherm of 4-5 °C was noted.
2. HPLC used for IPC and purity.
   Vydac C8, 260 nm
   1 mL/min, 262 nm, 30 °C
   A water/0.1% TFA

B 80:20 IPA/acetonitrile/0.1 % TFA
60-95%B/30 minutes.

3. The product precipitates with a fine particle size resulting in a slow filtration.
4. The material must be free of water for the heptane to effectively remove the dibenzylfulvene.

11.9 Large Scale Preparation of Fragment AcAA(1-36)-OH by Solution Phase Synthesis from AcAA(1-16)-OH and FmocAA(17-36)-OH

The AcAA1-16OH was dissolved in DMF with HOAT and DIEA, cooled to 0°C and HBTU was added. This was stirred for 15 minutes, or until the HBTU dissolved, then HAA17-36NH_2 was added. Pre-activation of AcAA1-16OH in the absence of HAA17-36NH_2 was a precaution that later was found not to be necessary. In addition, dissolution of HAA17-36NH_2 into the 0°C DMF solution containing the activated AcAA1-16OH proved to be slow on larger scale.

A total of 6.972 Kg of AcAA1-36NH_2 was prepared from 3.92 Kg of AcAA1-16OH and 4.96 Kg of HAA17-36NH_2 in four runs averaging 80.1% yield. Two runs in this stage averaged 87% yield and two runs averaged 73% yield.

A use test is run on a 1-2 g scale testing both fragments and the HBTU to be used prior to running the coupling reaction. The stoichiometry of starting materials and reagents is determined from the use test. The solubility of the starting materials is also noted in the use test. A cloudy solution may indicate the presence of salts in AcAA1-16OH and warrant a water wash on the fragment. It is imperative that all components of the reaction mixture are clearly in solution prior to addition of the HBTU to ensure complete conversion of the starting materials to product with minimal racemization and byproduct formation.

The fragments, AcAA1-16OH and HAA17-36NH_2, and reagents HOAT and DIEA were dissolved in DMF, cooled to 0 °C and HBTU is added. One of the runs required an additional equivalent of DIEA to pick up the HCl on HAA17-36NH_2. The crude product

is isolated from the reaction mixture with a water dropout (filtration time, 10 minutes). The still damp solid was returned to a reactor containing acetonitrile pre-warmed to 55 °C, then stirred rapidly while cooling to 35 °C over 3 hours, then to 20 °C over night. The slurry is cooled to 0-5 °C, stirred an additional 1-2 hours and the solid is collected by filtration. During this process, the AcAA1-36NH_2 nearly goes into solution at 55 °C. As the solution cools, the AcAA1-36NH_2 precipitates (oils) out of solution. As the solution cools, the oil solidifies and eventually is transformed into a nice white solid. During the course of this transformation, considerable deposition of solid on the reactor wall and stir shaft may occur. Most of this falls off as the slurry is stirred overnight at 20°C. After collecting the solid, the reactor is filled with enough water to cover any solid sticking to the reactor walls or shaft. The slurry is stirred until all remaining solid has fallen off the walls and shaft (approximately 1 hour), then used as a wash on the filter cake. The acetonitrile wash removes unreacted starting materials and any truncated fragments less than 20 amino acids.

11.9.1 Preferred Large Scale Preparation of Fragment AcAA(1-36)-OH by Solution Phase Synthesis from AcAA(1-16)-OH and FmocAA(17-36)-OH

A 40 liter reactor is charged with AcAA1-16OH (938 g, 1 eq), HAA17-36NH_2 (1.19 Kg, 1 eq.), HOAT (1 eq), DMF (19 vol, relative to AcAA1-16OH) and DIEA (1 eq.). The slurry is stirred until the solids dissolve, then cooled to 0-5 °C and HBTU (1.03 eq.) is added. The solution is stirred at 0-5 °C for 15 minutes or until the solid dissolves, warmed to 20 °C and stirred for 2 hours. The reaction mixture is sampled for an IPC (note 1). When the reaction is judged complete, water (19 vol) is added at such a rate as to not exceed 35 °C (note 2).

The slurry is stirred for 1-24 hours then the solid is collected by vacuum filtration (10 minutes) and washed with water (2 x 3 vol.). The filter cake is compressed to remove as much water as possible. Meanwhile, the reactor is charged with 95% acetonitrile/water (30 volumes relative to the AcAA1-16OH charge) and warmed to 55 °C with stirring.

The damp solid is added to the reactor in portions to avoid clumping. The slurry is stirred while cooling to 35 °C over 3 hours, then to 20 °C overnight. Cool to 0-5 °C, stir for 2 hours, stop stirring and sample the solution.

Collect the solid by vacuum filtration. Wash the reactor and lines with 90% acetonitrile/water (3.6 vol).

Charge the reactor with a sufficient amount of water to cover any solids sticking to the reactor walls or stir shaft (approximately 30 vol). Stir at ambient temperature until the solids are no longer sticking to the reactor walls and shaft (approximately 1 hour). Collect the solid with the rest and dry to constant weight (1.83 Kg, 86% yield).

Repeat trituration on the dry solid using 90/10 acetonitrile/water.

Notes:

1. IPC and purity, HPLC
   YMC ODS-A, 150 x 4.6 mm, 5 µ, 120A
   1 mL/min, 260 nm, 30 °C
   A water/0.1% TFA
   B THF/0.1% TFA
   60-90%B/30 min., 90-95%B/1 min., 95%B/5 min.
   AcAA1-16OH 6.37 minutes
   HAA17-36NH_2 8.91 minutes
   AcAA1-36NH_2 18.07 minutes
2. If the temperature exceeds 35 °C during the water addition, the solids that precipitate out can melt leading to clumps and/or deposition on the reactor walls.

11.10 Deprotection of Side Chains of AcAA(1-36)-OH

It was the objective of this experiment to change the addition-rate of MTBE in the precipitation to keep the temperature less than 20°C and also isolate and decarboxylate crude T-20 solid (i.e. remove solution decarboxylation).

The crude T20 was isolated by precipitation from ACN/water as a solid prior to loading onto an HPLC column. The percent T20 content of the solid was determined by a weight based HPLC assay.

After the side-chain protecting groups have been removed in the 90/5/5 TFA/water/dithiothreitol, the solution is cooled to 0 °C and MTBE (45 vol. relative to AcAA1-36NH_2 charge) is added. This is a minimum amount of MTBE required to ensure complete precipitation of T-20. There is an exotherm on mixing and the first 5 volumes must be added slowly to keep the temperature below 20 °C (approximately 60 minutes). As more MTBE is added, the rate of addition can be increased. Keeping the temperature below 20 °C during the precipitation prevents clumping of the solid as it precipitates out of solution.

The current cleavage/purification protocol involves running the deprotection on scale that accommodates chromatographic purification. The crude T-20 isolated from the deprotection cocktail as a TFA salt was dissolved in acetonitrile/water at pH 5, filtered and allowed to stand for 15 hours to effect decarboxylation of the tryptophan indoles. After the decarboxylation was complete, the solution was diluted and transferred to the purification suite to be loaded onto the column. During the dilution, the solution always became hazy and the turbid solution was pumped onto the column. This resulted in deposition of solid onto the head of the column and gradual erosion of column performance during the purification runs. Allowing T-20 to decarboxylate in acetonitrile/water at pH 5 for several hours leads to formation of highly insoluble aggregates.

A method to avoid decarboxylation in solution has been developed. The crude T-20, isolated as a TFA salt from the MTBE precipitation, will decarboxylate under vacuum at room temperature over about 5-7 days. The decarboxylation was found to be complete in three days at 40°C under vacuum. The TFA content of the solid isolated was 9%. The material can be stored for up to one month at 0°C with 1% wt/wt loss.

A salt exchange in ethanol/water (1:9) with HOAc can be performed after isolating T-20-TFA from MTBE. The acetate salt of crude T-20 is significantly more stable and may be preferred. Either isolation method will allow the side-chain deprotection to be run on larger scale and the T-20 will not be exposed to the acetonitrile/water/HOAc conditions used for decarboxylation that are known to cause aggregation.

New Method For Deprotection of Side Chains of AcAA(1-36)OH:

Prepare a 90/5/5 TFA/water/dithiothreitol solution (13 vol) and purge with nitrogen for 3 minutes. AcAA1-36NH_2 is added in portions to 90/5/5 TFA/water/dithiothreitol (13 vol) at ambient temperature under an atmosphere of nitrogen. Once in solution, stir at ambient temperature for 4 hours then cool to 0 °C. To precipitate the T-20, MTBE (45 vol), cooled to 0-5 °C, is added drop-wise at a slow rate initially maintaining a temperature below 20 °C (approximately 1-2 hour addition time). The solid is collected by vacuum filtration (note 1). The reactor, lines and filter cake are immediately washed with 3 x 5 volumes of MTBE. The solid is removed, sampled for t = 0 IPC (note 2) and dried under vacuum for 5 days, or until HPLC shows no change.

Notes:

1. Avoid pulling air through the solid during this filtration as the deprotection solution is hygroscopic. Pulling air through solid before the TFA solution has been washed out can result in a tacky or oily solid.

2. Use HPLC method TM2-0003-01 (TFA method). The ammonium acetate method (TM2-0006-01) does not separate the various carboxylated intermediates.

<u>Deprotection of Side Chains of AcAA(1-36)OH</u>

A total of 7.226 Kg of AcAA1-36NH_2 was deprotected in 12 runs. Only 6.972Kg of AcAA1-36NH_2 was prepared suggesting that the intermediate may be hygroscopic. The solid isolated from the MTBE dropout was dissolved in 10 volumes of 1:1 ACN/water, filtered and adjusted to pH 3.5 with sodium bicarbonate. Acetic acid (1.5 volume %) was added (pH 4.5 to 5) and the solution was stirred at ambient temperature for 15 hours to effect decarboxylation. The solution was diluted with 25 volumes of water give a total of 40 volumes of an 85/15 water/ACN solution. The solution was turbid in all cases and in some, had a fine solid (aggregated T-20). A sample was taken for IPC and the solution was transferred to the purification suite. Crude T-20 yields for the runs were calculated using a wt/wt HPLC assay.

The deprotection of AcAA1-36NH_2 is complete in 90/5/5 TFA/water/DTT at room temperature after 4-5 hours. At 8 hours, noticeable degradation (2%) has occurred. In the same cocktail at 5°C, the deprotection is not complete at 8 hours, but is at 23 hours. There is no difference in the purity of the crude T-20 obtained after 5 hours at room temperature versus 23 hours at 5°C. Because the isolation volume (approximately 55 vol) is much larger then the reaction (deprotection) volume (13 vol), it was necessary to dissolve the AcAA1-36NH_2 in the TFA solution in a 20L carboy, then transfer the solution to a 40L reactor.

Isolation of the crude, carboxylated T-20 (as a TFA salt) from the deprotection cocktail is accomplished by precipitation with MTBE. The amount of MTBE needed to precipitate the peptide is 3-4 times the amount of deprotection cocktail. Using MTBE at twice the volume (or less) of the cleavage cocktail leaves peptide behind.

Addition of the MTBE to the cleavage cocktail results in an easily filtered solid while adding the cocktail to MTBE produces a fine precipitate which is difficult to filter. There is a temperature rise from 5°C to 40-45°C during the addition of MTBE to the TFA solution. The temperature increase has no effect on the purity of the crude T-20 isolated (187/117,119). There was some clumping of the solids during the MTBE addition. The slurry was stirred for 1-2 hours to break up the clumps. Maintaining a temperature below 20°C during the MTBE addition produces a more uniform solid that filters better (196/31). The addition rate of the MTBE will be controlled in the future. Crude T-20 was collected by filtration under a nitrogen atmosphere. The solid will become tacky if exposed to moist air during the filtration before the TFA has been washed out. After the TFA is washed out, the solid is air stable.

Method Run:

A 90/5/5 TFA/water/dithiothreitol solution (13 vol) is prepared in a 20 L carboy then purged with nitrogen for 3 minutes. AcAA1-36NH_2 (650 g) is added in portions to prevent clumping. Once the solid is in solution, the solution is transferred to a 70L reactor. The carboy and lines are washed with a minimal amount of 90/5/5/TFA/water/ dithiothreitol. The solution is stirred at ambient temperature under an atmosphere of nitrogen for 4 hours, then cooled to 0-5 °C. MTBE (45 vol) is added at a rate such that the internal temperature is less than 30 °C. The solid is collected by vacuum filtration under a stream of nitrogen. The reactor, lines and solid are washed with 2 x 2 volumes of MTBE and dried to constant weight (594 g, 70A%).

The solid (594 g) is dissolved in 50% ACN/water (8 volumes relative to the AcAA1-36H_2 charge) and filtered. The vessel and lines are washed with 50% ACN/water (2 vol). The pH of the solution is adjusted to 3.5-4.0 with sodium bicarbonate, then acetic acid (0.18 vol) is added bringing

the pH to 4.9. The solution is stirred at ambient temperature for 15 hours (IPC, note 1), then the pH is adjusted to 9-9.5 with 1 M potassium carbonate. The solution is diluted to 85/15 water/ACN by adding water (25 vol). The resulting turbid solution is sampled for wt/wt HPLC analysis and transferred to the purification suite.

Notes:

1 Method TM2-0003-01 .

2 Method TM2-0006-01 .

11.11 Purification of HAA(1-36)-OH

Purification, lyophilization and packaging are classified in three stages:

%wt/wt = 100 - % impurities - % acetate - % water - % TFA. The acetate content in the batches was 6-8%.

A total of 2.08 Kg of T-20 (net) was isolated from 6.97 Kg of AcAA1-36NH_2. This represents a 49.3% yield from AcAA1-36NH_2. The average yield in the chromatographic purification of the was 55%. The yields for individual runs varied from 41-55%. The lowest yields were a result of column or pump failures leading to multiple passes. In general, if the chromatography worked, the yields were in the 50-55% range. The purity of the T-20 isolated was 93-95A%.

Four gradients were examined during purification for a comparison of methods with the objective of minimizing the run time:

1. 15-22% ACN/60 minutes, 22-36% ACN/525 minutes at 330 mL/min.
2. 16-26% ACN/60 minutes, 26-40% ACN/525 minutes at 330 mL/min.
3. Second Trimeris gradient. 15-36% ACN1788 minutes at 330 mL/min.
4. Original Trimeris gradient 20-23% ACN/1 12 minutes, 23-36% ACN/488 minutes at 330 mL/min.

A twenty centimeter (cm) column was packed (axial compression) with the Amberchrom resin (35 cm bed height). Batches of 500-700g AcAA1-36NH2 were deprotected and decarboxylated in solution. This scale produces a column feed stock that contains approximately 400 grams of peptide (approximately 75A% T-20). The stock solution is typically hazy and may contain suspended solid. The turbid solution is loaded onto the column at 500 mL/min using 15% B. There was no pressure build up during the column load for any of the runs. The flow rate is reduced to 330 mL/min and the specified gradient is run for the indicated amount of time. Fractions are collected and those containing over 78% T-20 are pooled (100-110L), diluted with water to bring the acetonitrile content to approximately 15-20% (approximately 140 liters). The column is washed, then equilibrated with 15% B. The T-20 containing solution is loaded back onto the 8 inch column at 900 mL/min. The %B is increased to 50% and T-20 is flushed of the column in approximately 25L.

The solution is frozen in one liter bell jars and lyophilized to a powder. The T20 isolated typically is 92-94A% by HPLC, and contains 6-8% acetate and 3-4% water.

Preferred Method:

A solution of T-20 in 85/15 water/acetonitrile (27L) at pH 9.2 was pumped onto an eight inch HPLC column at 500 mL/min. The Flow rate was reduced to 330 mL/min over 30 minutes (in increments of 30-40 mL/min every 5 minutes). A linear gradient going from 20%B to 36%B over 600 minutes is initiated. Fractions are collected until absorbance falls below 0.2 AUFS. The fractions are analyzed by HPLC (note 1) for content. The column is washed with 80%B at 900 mL/min for 10 minutes, then brought back to 15%B and equilibrated at 900 mL/min. The fractions containing over 78A% T-20 are pooled (113 L) and diluted with buffer (28.2 L) to bring the acetonitrile concentration to 15-20%. The solution is pumped onto the column at 900 mL/min. The %B is increased to 50%

and T-20 is eluted off the column at 900 mL/min in approximately 25L. The concentration of T-20 is approximately 9-10 mg/mL. The solution is transferred to one liter lyophilization jars, frozen and freeze dried to give 216.29 of T-20 in 54.9% yield and 94.1A% purity.

Notes:

1. Fractions are analyzed by HPLC for content and purity.
   YMC ODS-A, 150 x 4.6 mm, 5 µm, 120A
   1 mL/min, 220 nm, 30°C
   A 70/30 50 mM NH_4OAc @ pH 7 (adjust with NH_4OH):ACN
   B 5:95 50 mM NH_4OAc @ pH 7 (adjust with NH_4OH):ACN
   0-15%B/50 min., 15-100%B/2 min. 100%B/3 min.
   T-20 retention time, 29.0 minutes.

11.12 Thermal Stability of Peptides

A series of experiments were conducted at to characterize the thermal stability of the intermediate T-20 fragments. T-20 fragments were saturated with water, filtered and then placed in enclosed containers and exposed to elevated temperatures. At several time points samples were collected and analyzed by HPLC methods to access the purity changes. Stability of the dry T-20 fragments was also characterized by thermogravimetric analysis (TGA).

All of the fragments can be dried at 40 °C for 3 days but AcAA1-16OH. Only modest degradation is observed after 24 hours at 80 °C in two fragments, FmocAA27-35OH (3%) and FmocAA27-36NH_2 (1.4%). Fragment AcAA1-16OH was initially 97A% pure. After 3 days at 40 °C, it was 93.4A% pure and dry. After an additional 24 hours at 80 °C it remained at 93.9A%. The damp AcAA1-16OH should be dried at ambient temperature.

The point of the stability study was to determine if the fragments can be dried at 40 °C rather then ambient temperature. The study indicates that the fragments are stable to drying at 40 °C. This will greatly reduce the drying times.

A study designed to examine the stability of fragments Ac(1-16)-OH, Fmoc(27-35)-OH, Fmoc(17-26)-OH, Fmoc(27-36)-NH_2, Fmoc(17-36)-NH_2, H(17-36)-NH_2, and Ac(1-36)-NH_2 under various solvent and temperature conditions that mimic their isolation and purification has been completed. All of the fragments are stable, stability is designated with an X, in the solvent systems from which they are isolated and/or purified:

		Time				
Intermediate	Storage solution	Initial	1 hour	6 hour	1 day	3 day
F(27-35)-OH	9:1 EtOH:$H_2$0, 40°C	X	X	X	X	X
F(17-26)-OH	80:20 EtOH/$H_2$0, 40°C	X	X	X	X	X
F(27-36)-NH_2	1:1 NMP/$H_2$0, 40°C	X	X	X	X	X
Ac(1-16)-OH	0.01 M HCl, 5-10°C	X	X	X	X	X
F(17-36)-NH_2	95% IPA/$H_2$0, 60-65°C	X	X	X	X	X
	50%$H_2$0/NMP, 50°C	X	X	X	X	X
	90:10 EtOH/$H_2$0, 40°C	X	X	X	X	X
H(17-36)-NH_2	1.5:1 H20/DMF, 40°C	X	X	X	X	X
Ac(1-36)-NH_2	9:1 ACN/$H_2$0, 50°C	X	X	X	X	X
	50%$H_2$0/NMP, 50°C	X	X	X	X	X
	85:15 EtOH:$H_2$0, 70°C	X	X	X	X	X

X = HPLC content and impurities, visual appearance check

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. A method for the synthesis of a peptide having the formula Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1), comprising:

(a) reacting a side-chain protected peptide of the formula: Fmoc-EKNEQELLEL-COOH (SEQ ID NO:11) with a side chain-protected peptide of the formula:

NH_2-DKWASLWNWF-NH_2 (SEQ ID NO:18)

to yield a side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12);

(b) deprotecting the amino terminus of the peptide produced in (a);

(c) reacting the peptide produced in (b) with a side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4)

to yield a side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1);

(d) modifying the amino terminus of the peptide produced in (c) into an acetyl modification; and

(e) deprotecting the side chains of the side-chain protected peptide of (d) to yield a peptide of the formula: Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1).

2. A method for the synthesis of a peptide having the formula Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1), comprising:

(a) reacting a side-chain protected peptide of the formula: Fmoc-EKNEQELLEL-COOH (SEQ ID NO:11) with a side chain-protected peptide of the formula:

NH_2-DKWASLWNWF-NH_2 (SEQ ID NO:18)

to yield a side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12);

(b) deprotecting the amino terminus of the peptide produced in (a);

(c) reacting the peptide produced in (b) with a side-chain protected peptide of the formula:

Ac-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4)

to yield a side-chain protected peptide of the formula:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1); and

(d) deprotecting the side chains of the side-chain protected peptide of (c) to yield a peptide of the formula: Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1).

3. A method for the synthesis of a peptide having the formula:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1), comprising:

(a) deprotecting the amino terminus of a side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12);

(b) reacting the peptide produced in (a) with a side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4),

to yield a side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1);

(c) deprotecting the amino terminus of the peptide produced in (b);

(d) modifying the amino terminus of the peptide produced in (c) into an acetyl modification; and

(e) deprotecting the side chains of the side-chain protected peptide of (c) to yield a peptide of the formula:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1).

4. A method for the synthesis of a peptide having the formula:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1), comprising:

(a) deprotecting the amino terminus of a side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12);

(b) reacting the peptide produced in (a) with a side chain protected peptide of the formula:

Ac-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4),

to yield a side-chain protected peptide of the formula:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1);

(c) deprotecting the side chains of the side-chain protected peptide of (b) to yield a peptide of the formula:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1).

5. The method of claim 3 or 4 wherein the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)

is synthesized by a method comprising:

(a) reacting a side-chain protected peptide of the formula:

Fmoc-DKWASLWNW-COOH (SEQ ID NO:17)

with $HPheNH_2$ to yield a side-chain protected peptide of the formula:

Fmoc-DKWASLWNWF-NH_2 (SEQ ID NO:18);

(b) deprotecting the amino terminus of the peptide produced in (a); and

(c) reacting the peptide produced in (b) with a side-chain protected peptide of the formula:

Fmoc-EKNEQELLEL-COOH (SEQ ID NO:11)

to yield the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12).

6. The method of claim 3 or 4 wherein the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)

is synthesized by a method comprising:

(a) reacting a side-chain protected peptide of the formula:

Fmoc-LELDKWASLWNW-COOH (SEQ ID NO:15)

with $HPheNH_2$ to yield a side-chain protected peptide of the formula:

Fmoc-LELDKWASLWNWF-NH_2 (SEQ ID NO:16);

(b) deprotecting the amino terminus of the peptide produced in (a); and

(c) reacting the peptide produced in (b) with a side-chain protected peptide of the formula:

Fmoc-EKNEQEL-COOH (SEQ ID NO:10)

to yield the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12).

7. The method of claim 3 or 4 wherein the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)

is synthesized by a method comprising:

(a) deprotecting the amino terminus of the peptide:

Fmoc-DKWASLWNWF-COOH (SEQ ID NO:16); and

(b) reacting the peptide produced in (a) with a side-chain protected peptide of the formula:

Fmoc-EKNEQELLEL-COOH (SEQ ID NO:10)

to yield the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12).

8. The method of claim 3 or 4 wherein the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)

is synthesized by a method comprising:

(a) reacting a side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNW-COOH (SEQ ID NO:19)

with $HPheNH_2$ to yield the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12).

9. The method of Claim 1 or 3 wherein the side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4)

is synthesized by a method comprising:

(a) deprotecting the amino terminus of the side-chain protected peptide of the formula:

Fmoc-IEESQNQQ-NH_2 (SEQ ID NO:7);

(b) reacting the side-chain protected peptide produced in (a) with a side-chain protected peptide of the formula:

Fmoc-YTSLIHSL-COOH (SEQ ID NO:2)

to yield the side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4).

10. The method of Claim 2 or 4 wherein the side-chain protected peptide of the formula:

Ac-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4)

is synthesized by a method comprising:

(a) reacting a side-chain protected peptide of the formula:

Fmoc-IEESQNQ-NH_2 (SEQ ID NO:6).

with HGlnOPNB to yield a side-chain protected peptide of the formula:

Fmoc-IEESQNQQ-OPNB (SEQ ID NO:7);

(b) deprotecting the amino terminus of the side-chain protected peptide produced in (a);

(c) reacting the side-chain protected peptide produced in (b) with a side-chain protected peptide of the formula:

Ac-YTSLIHSL-COOH (SEQ ID NO:2)

to yield a side-chain protected peptide of the formula:

Ac-YTSLIHSLIEESQNQQ-OPNB (SEQ ID NO:4);

(d) deprotecting the carboxyl terminus of the side-chain protected peptide produced in (c) to yield the side-chain protected peptide of the formula:

Ac-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4).

11. The method of claim 1 or 3 wherein the side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4)

is synthesized by a method comprising:

(a) reacting a side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQ-COOH (SEQ ID NO:3)

with HGlnOPNB to yield the side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQ-OPNB (SEQ ID NO:4); and

(b) deprotecting the carboxyl terminus of the peptide produced in (a) to yield the side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQ-COOH (SEQ ID NO:4).

12. A method for the synthesis of a peptide of the formula:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1), comprising:

(a) deprotecting the amino terminus of a side-chain protected peptide of the formula:

Fmoc-QEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:9);

(b) reacting the peptide produced in (a) with a side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQ-COOH (SEQ ID NO:3),

to yield a side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1);

(c) modifying the amino terminus of the peptide produced in (b) into an acetyl modification; and

(d) deprotecting the side chains of the side-chain protected peptide of (c) to yield the peptide of the formula:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1).

13. The method of claim 12 wherein the side-chain protected peptide of the formula:

Fmoc-QEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:9),

is synthesized by a method comprising:

(a) reacting a side-chain protected peptide of the formula:

Fmoc-QEKNEQELLELDKWASLWNW-NH_2 (SEQ ID NO:8)

with HPheNH_2 to yield the side-chain protected peptide of the formula:

Fmoc-QEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:9).

14. A method for the synthesis of a peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1), comprising:

(a) deprotecting the amino terminus of a side-chain protected peptide of the formula:

Fmoc-NEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:14);

(b) reacting the peptide produced in (a) with a side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQEK-COOH (SEQ ID NO:5),

to yield a side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1);

(c) modifying the amino terminus of the peptide produced in (b) into an acetyl modification; and

(d) deprotecting the side chains of the side-chain protected peptide of (c) to yield the peptide of the formula:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1).

15. The method of Claim 14 wherein the side-chain protected peptide of the formula:

Fmoc-NEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:14),

is synthesized by a method comprising:

(a) reacting a side-chain protected peptide of the formula:

Fmoc-NEQELLELDKWASLWNW-NH_2 (SEQ ID NO:13)

with $HPheNH_2$ to yield the side-chain protected peptide of the formula:

Fmoc-NEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:14).

16. The method of claim 1, 2, 3, 4, 12, or 14 wherein the side-chain protected peptide of the formula:

Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:1),

is produced in an amount of about one or more kilograms.

17. The method of claim 1, 2, 3, or 4 wherein the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)

is produced in an amount of about one or more kilograms.

18. The method of claim 5 wherein the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)

is produced in an amount of about one or more kilograms.

19. The method of claim 6 wherein the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)

is produced in an amount of about one or more kilograms.

20. The method of claim 7 wherein the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)

is produced in an amount of about one or more kilograms.

21. The method of claim 8 wherein the side-chain protected peptide of the formula:

Fmoc-EKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:12)

is produced in an amount of about one or more kilograms.

22. The method of claim 9 wherein the side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQ-NH_2 (SEQ ID NO:4)

is produced in an amount of about one or more kilograms.

23. The method of claim 10 wherein the side-chain protected peptide of the formula:

Ac-YTSLIHSLIEESQNQQ (SEQ ID NO:4)

is produced in an amount of about one or more kilograms.

24. The method of claim 11 wherein the side-chain protected peptide of the formula:

Fmoc-YTSLIHSLIEESQNQQ-NH_2 (SEQ ID NO:4)

is produced in an amount of about one or more kilograms.

25. The method of claim 13 wherein the side-chain protected peptide of the formula:

Fmoc-QEKNEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:9)

is produced in an amount of about one or more kilograms.

26. The method of claim 15 wherein the side-chain protected peptide of the formula:

Fmoc-NEQELLELDKWASLWNWF-NH_2 (SEQ ID NO:14)

is produced in an amount of about one or more kilograms.

27. A set of peptide fragments comprising a set selected from the group consisting of:

(a) YTSLIHSLIEESQNQQ (SEQ ID NO:4),
EKNEQELLELDKWASLWNWF (SEQ ID NO:12);

(b) YTSLIHSLIEESQNQQ, (SEQ ID NO:4),
EKNEQELLEL (SEQ ID NO:11),
DKWASLWNWF (SEQ ID NO:18);

(c) YTSLIHSLIEESQNQQ (SEQ ID NO:4),
EKNEQELLEL, (SEQ ID NO:11),

DKWASLWNW (SEQ ID NO:17);

(d) YTSLIHSL (SEQ ID NO:2),
IEESQNQ (SEQ ID NO:6),
EKNEQELLELDKWASLWNWF (SEQ ID NO:12);

(e) YTSLIHSL (SEQ ID NO:2),
IEESQNQ (SEQ ID NO:6),
EKNEQELLEL (SEQ ID NO:11),
DKWASLWNWF (SEQ ID NO:18);

(f) YTSLIHSL (SEQ ID NO:2),
IEESQNQ (SEQ ID NO:6),
EKNEQELLEL (SEQ ID NO:11),
DKWASLWNW (SEQ ID NO:17);

(g) YTSLIHSL (SEQ ID NO:2),
IEESQNQQ (SEQ ID NO:7),
EKNEQELLELDKWASLWNWF (SEQ ID NO:12);

(h) YTSLIHSL (SEQ ID NO:2),
IEESQNQQ (SEQ ID NO:7),
EKNEQELLEL (SEQ ID NO:11),
DKWASLWNWF (SEQ ID NO:18);

(i) YTSLIHSL (SEQ ID NO:2),
IEESQNQQ (SEQ ID NO:7),
EKNEQELLEL (SEQ ID NO:11),
DKWASLWNW (SEQ ID NO:17);

(j) YTSLIHSLIEESQNQQ (SEQ ID NO:4),
EKNEQEL (SEQ ID NO:10),
LELDKWASLWNWF (SEQ ID NO:16);

(k) YTSLIHSLIEESQNQQ (SEQ ID NO:4),
EKNEQEL (SEQ ID NO:10),
LELDKWASLWNW (SEQ ID NO:15);

(l) YTSLIHSL (SEQ ID NO:2),
IEESQNQ (SEQ ID NO:6),
EKNEQEL (SEQ ID NO:10),
LELDKWASLWNWF (SEQ ID NO:16);

(m) YTSLIHSL (SEQ ID NO:2),
IEESQNQ (SEQ ID NO:6),
EKNEQEL (SEQ ID NO:10),
LELDKWASLWNW (SEQ ID NO:15);

(n) YTSLIHSL (SEQ ID NO:2),
IEESQNQQ (SEQ ID NO:7),
EKNEQEL (SEQ ID NO:10),
LELDKWASLWNWF (SEQ ID NO:16);

(o) YTSLIHSL (SEQ ID NO:2),

IEESQNQQ (SEQ ID NO:7), EKNEQEL (SEQ ID NO:10), LELDKWASLWNW (SEQ ID NO:15);

(p) YTSLIHSLIEESQNQ (SEQ ID NO:3), QEKNEQELLELDKWASLWNW (SEQ ID NO:8);

(q) YTSLIHSLIEESQNQ (SEQ ID NO:3), QEKNEQELLELDKWASLWNWF (SEQ ID NO:9);

(r) YTSLIHSLIEESQNQQEK (SEQ ID NO:5), NEQELLELDKWASLWNWF (SEQ ID NO:14);

(s) YTSLIHSLIEESQNQQEK (SEQ ID NO:5), NEQELLELDKWASLWNW (SEQ ID NO:13); and

(t) YTSLIHSLIEESQNQQ (SEQ ID NO:4), EKNEQELLELDKWASLWNW (SEQ ID NO:19).

28. A peptide selected from the group consisting of:

YTSLIHSL (SEQ ID NO:2);
IEESQNQ (SEQ ID NO:6);
IEESQNQQ (SEQ ID NO:7);
QEKNEQELLELDKWASLWNW (SEQ ID NO:8);
EKNEQEL (SEQ ID NO:10);
EKNEQELLEL (SEQ ID NO:11);
NEQELLELDKWASLWNW (SEQ ID NO:13);
LELDKWASLWNW (SEQ ID NO:15);
LELDKWASLWNWF (SEQ ID NO:16);
DKWASLWNW (SEQ ID NO:17);
DKWASLWNWF (SEQ ID NO:18);
and
EKNEQELLELDKWASLWNW (SEQ ID NO:19).

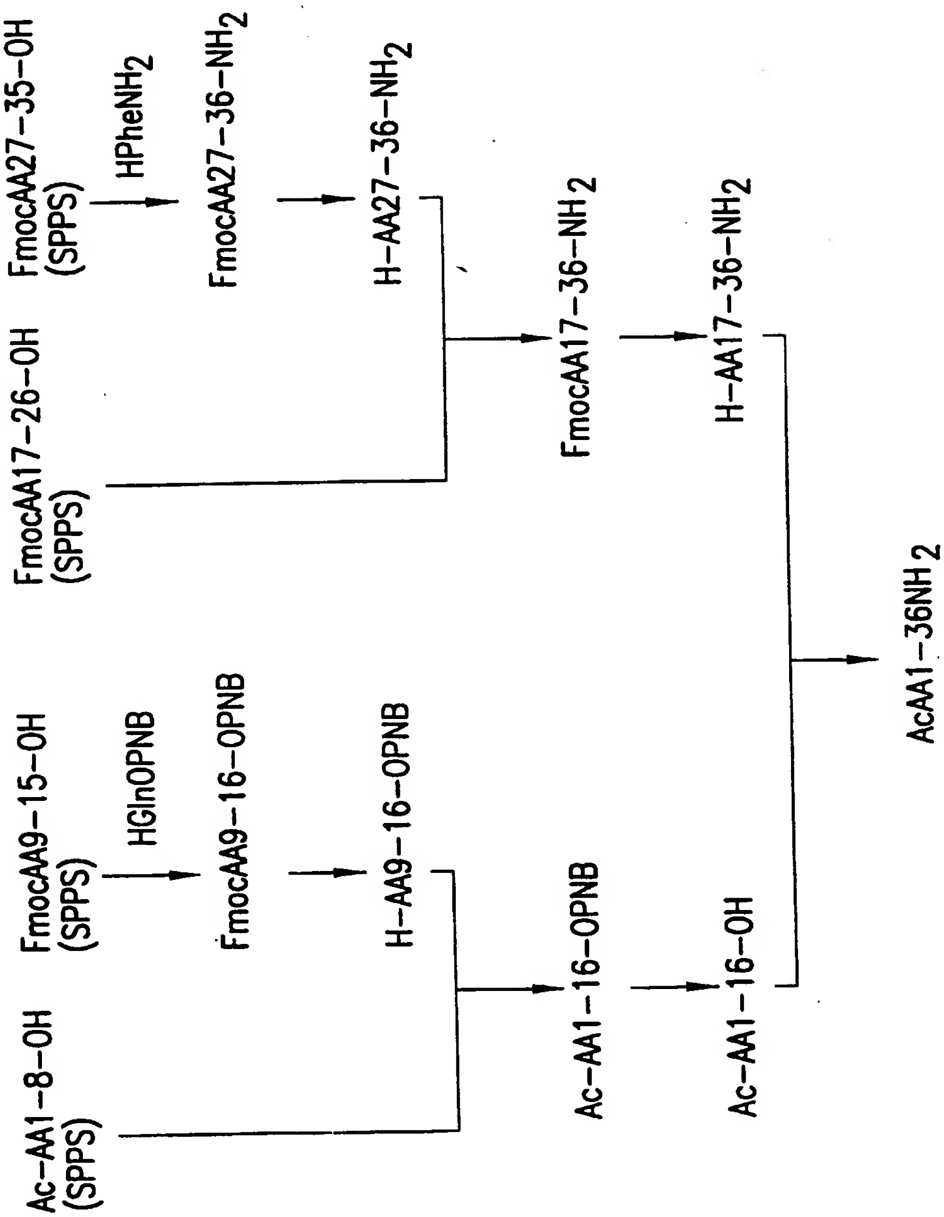
Ac–AA1–8–OH (SPPS)
FmocAA9–15–OH (SPPS)
HGlnOPNB
FmocAA9–16–OPNB
H–AA9–16–OPNB
Ac–AA1–16–OPNB
Ac–AA1–16–OH
FmocAA17–26–OH (SPPS)
FmocAA27–35–OH (SPPS)
HPheNH2
FmocAA27–36–NH2
H–AA27–36–NH2
FmocAA17–36–NH2
H–AA17–36–NH2
AcAA1–36NH2

FIG. 1

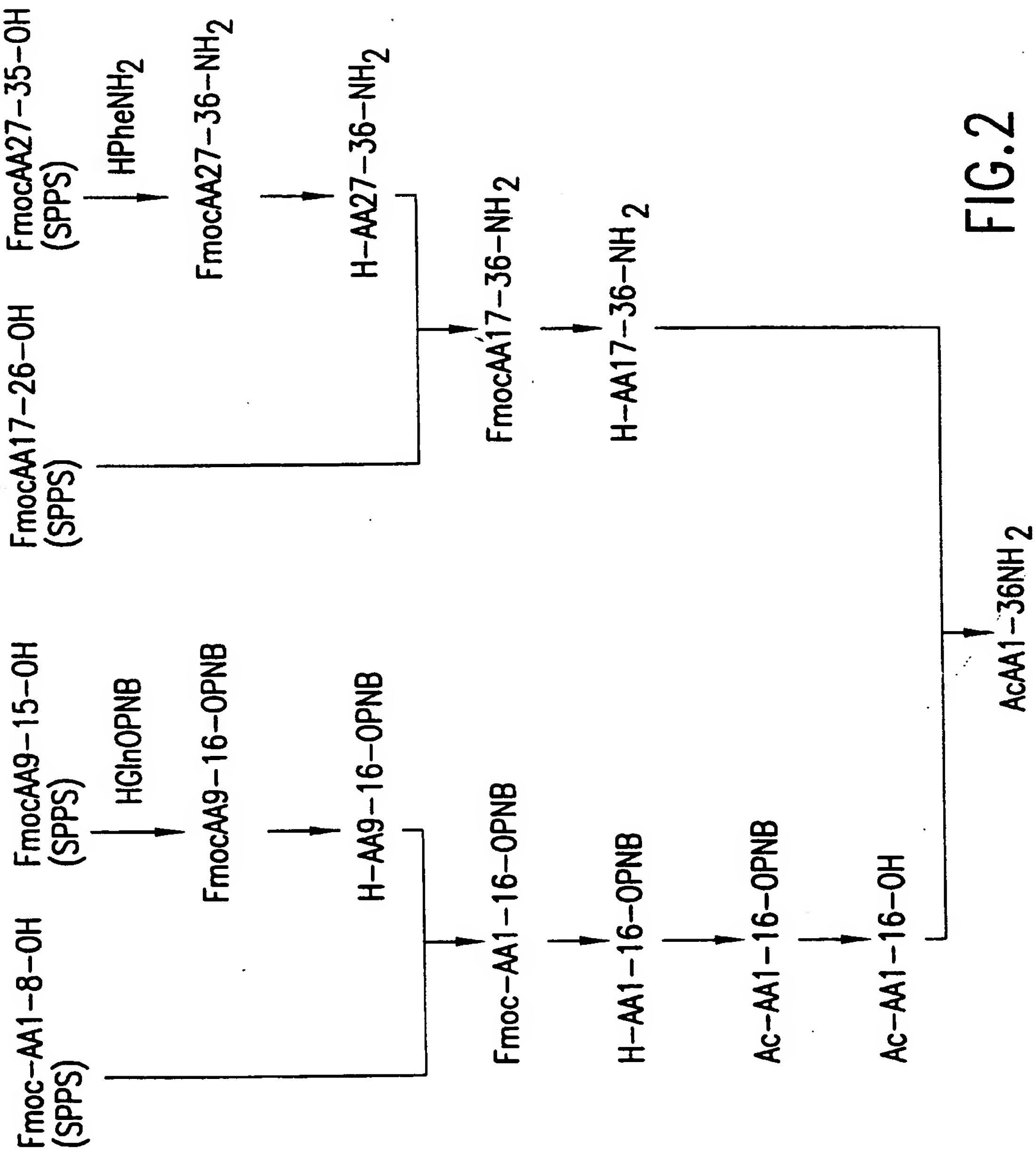
FmocAA27–35–OH (SPPS)
HPheNH$_2$
FmocAA27–36–NH$_2$
H–AA27–36–NH$_2$
FmocAA17–26–OH (SPPS)
FmocAA17–36–NH$_2$
H–AA17–36–NH$_2$
FmocAA9–15–OH (SPPS)
HGlnOPNB
FmocAA9–16–OPNB
H–AA9–16–OPNB
Fmoc–AA1–8–OH (SPPS)
Fmoc–AA1–16–OPNB
H–AA1–16–OPNB
Ac–AA1–16–OPNB
Ac–AA1–16–OH
AcAA1–36NH$_2$

FIG.2

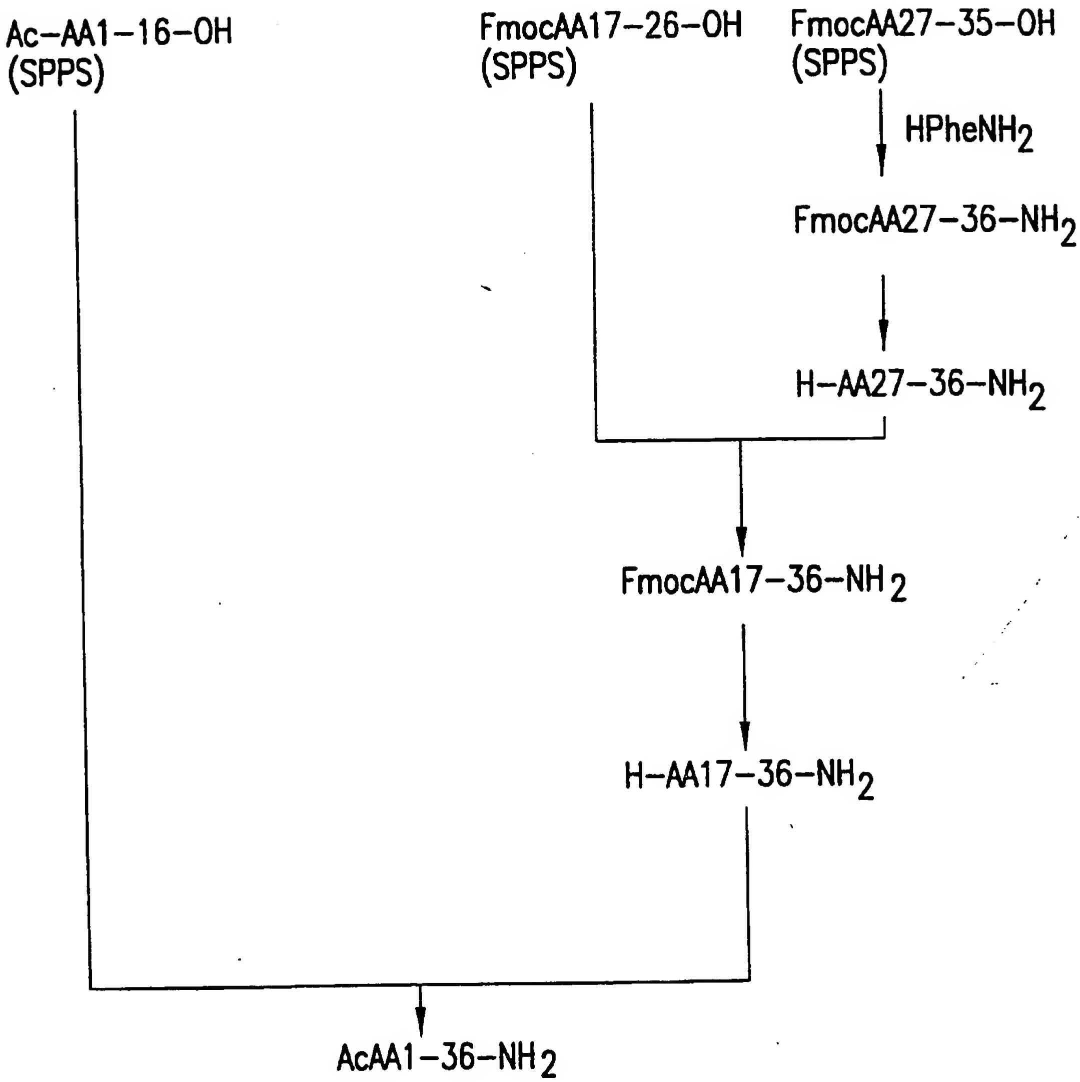
Ac–AA1–16–OH
(SPPS)
FmocAA17–26–OH
(SPPS)
FmocAA27–35–OH
(SPPS)
HPheNH2
FmocAA27–36–NH2
H–AA27–36–NH2
FmocAA17–36–NH2
H–AA17–36–NH2
AcAA1–36–NH2

FIG.3

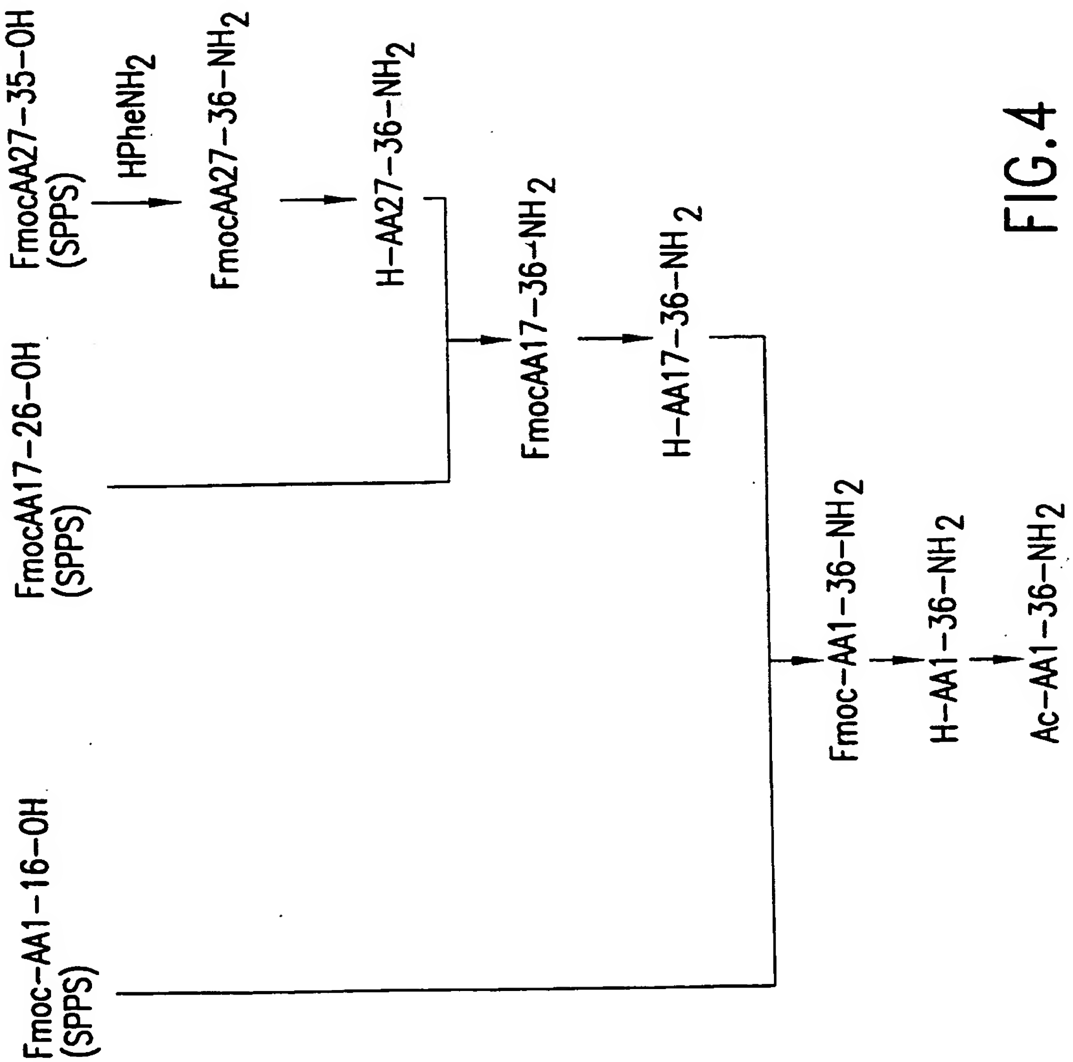
FmocAA27-35-OH
(SPPS)
HPheNH2
FmocAA27-36-NH2
H-AA27-36-NH2
FmocAA17-26-OH
(SPPS)
FmocAA17-36-NH2
H-AA17-36-NH2
Fmoc-AA1-16-OH
(SPPS)
Fmoc-AA1-36-NH2
H-AA1-36-NH2
Ac-AA1-36-NH2

FIG.4

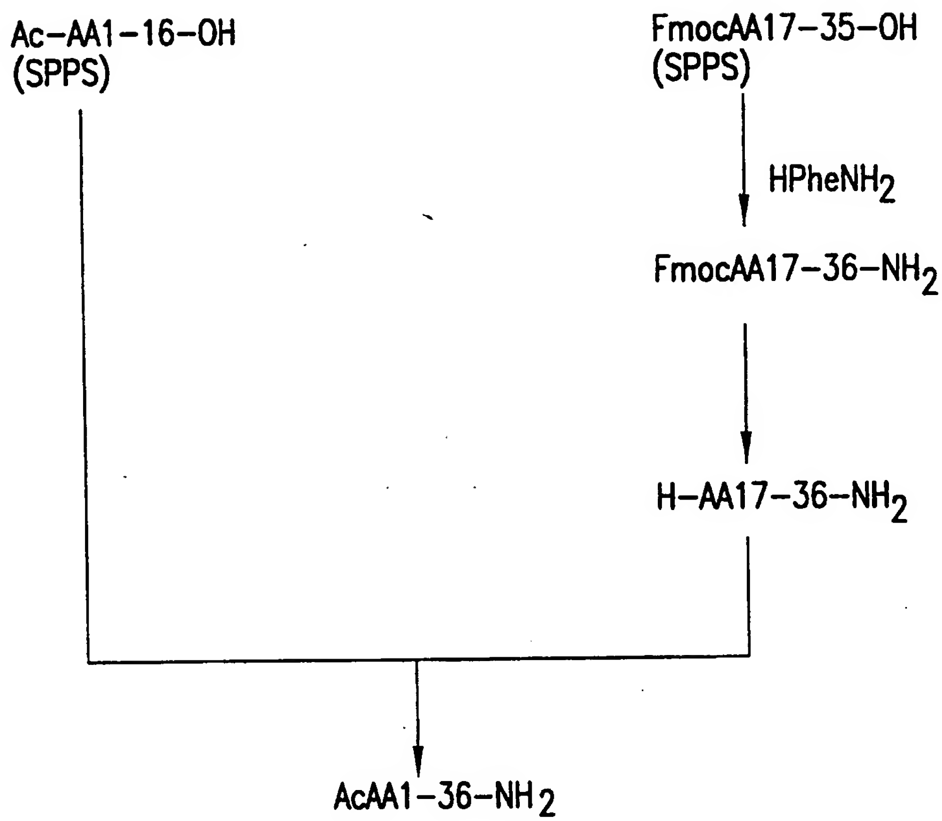
Ac-AA1-16-OH
(SPPS)
FmocAA17-35-OH
(SPPS)
HPheNH2
FmocAA17-36-NH2
H-AA17-36-NH2
AcAA1-36-NH2

FIG.5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06230

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00; C07K 5/00, 7/00, 16/00,17/00.
US CL : 514/12; 530/324, 333.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12; 530/324, 333.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS Registry, CAPLUS, search terms: fragment condens?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94/28929 A1 (DUKE UNIVERSITY) 22 December 1994, page 18, lines 11-12 and 14, page 19, lines13-14.	28

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 JUNE 1999

Date of mailing of the international search report

12 JUL 1999

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